IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.:	**09/911,692**	Group Art Unit:	1644
Confirmation No.:	8484	Examiner:	R. Schwadron
Filed:	25 July 2001		
Applicant:	Darrell R. ANDERSON et al.		
For:	Expression and Use of Anti-CD20 Antibodies (as amended)		

Mail Stop **AF**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MITCHELL E. REFF UNDER 37 C.F.R. § 1.132

1. I am the same Mitchell E. Reff named as an inventor in the patent application identified above. I am a citizen of the United States and reside in San Diego, California. A copy of my C.V. is attached as an exhibit to this declaration. I am currently employed by Biogen Idec Inc. as Vice President, Discovery Oncology.

2. I have reviewed the application identified above, the claims presented in the amendment filed 13 December 2006, and the final Office action dated 8 March 2007.

3. I have been asked to comment on whether this application would have described the following subject matter to a person skilled in the art in November 1992:

> A host cell comprising nucleic acid sequences encoding the light chain and the heavy chain of an immunologically active chimeric anti-CD20 antibody, wherein the sequence encoding the light chain comprises a nucleotide sequence encoding amino acid residues 23 to 128 of SEQ ID NO: 4, and the sequence encoding the heavy chain comprises a nucleotide sequence encoding amino acid residues 20 to 140 of SEQ ID NO: 6.

4. I understand that the standard for evaluating whether an application describes the subject matter of a claim is whether the disclosure "reasonably conveys to the artisan that the

inventor had possession" of that subject matter when the application was filed. I believe the specification would have informed a person skilled in this art that our invention included a host cell having the characteristics stated in paragraph 3 above.

5. I note first that nucleic acid sequences encoding antibodies having the structures specified in the claims are described throughout the application. Immunologically active chimeric anti-CD20 antibodies are described, for example, at page 13, lines 12-16, and page 8, lines 14-16. The amino acid sequences specified in the claims are illustrated in Figure 4 and Figure 5 (as corrected on 22 August 2005).

6. I understand that the nature of the "host cells" described in our application is at issue. I note that the application does not provide any definition of that term that would give it anything other than its ordinary meaning. Instead, "host cells" comprising the nucleic acid sequences are broadly described at page 19, line 22 to page 20, line 5, and at page 24, lines 15-26. I believe that a person skilled in the field of the invention reading the application in November 1992 would have understood the term "host cell" to refer to a cell into which exogenous nucleic acid had been introduced or, depending on the context, to a cell that would be suitable for such introduction.

7. One of the uses for host cells, discussed at page 24, line 1, is for "protein expression." However, I do not read the disclosure as limiting host cells to those transfected with an expression plasmid. As I explain below, other uses for host cells, such as for expanding nucleic acids, were known in the art in November 1992, and such uses are described in the application.

8. Our application describes several different ways for incorporating nucleic acids into host cells. For example, the application describes methods that "include, but are not limited to, transfection (including electrophoresis and electroporation), cell fusion with enveloped DNA, microinjection, and infection with intact virus." *See* page 24, lines 20-25. The preferred method described in the application is electroporation of the host cell with a plasmid, as noted at page 24, lines 25-26, and the use of this technique for introducing a plasmid expression vector encoding the heavy chain and light chain of a chimeric anti-CD20 antibody is described in an example at pages 40-43. However, the application unambiguously conveys that other methods for introducing nucleic acids into

host cells are contemplated, including those that are specifically identified (*e.g.*, cell fusion with enveloped DNA, microinjection, and infection with intact virus). One could have, for example, microinjected host cells with just the nucleic acid of interest.

9. In connection with a description of methods for introducing nucleic acids into host cells at page 24, the application cites a monograph chapter by Ridgway entitled "Mammalian Expression Vectors," published in 1988. A copy of this chapter is attached as an exhibit to this declaration. This chapter provides further detail about methods that were known and widely practiced by persons skilled in this field in November 1992 for introducing nucleic acids into host cells. The chapter begins by noting, at page 467, that the term "vector" in molecular biology had (as it still has) a "broad definition." As Ridgway explains, and as a person skilled in molecular biology would have immediately appreciated from the reference to the various techniques we identified in our application, the methods useful for introducing nucleic acids into host cells were not limited to transfection with a plasmid. *See* Ridgway at section 24.2, pages 470-472. As Ridgway demonstrates, a broad range of methods for introducing exogenous nucleic acids into host cells were very well known in the art by November 1992.

10. Host cells used for expanding nucleic acid sequences are described in the application. For example, the text at page 21, lines 22-24, describes the preparation of a nucleic acid sequence "prepared from RNA which was in turn derived from cells transfected with a human IgG1 vector." As persons skilled in the art understood, such host cells did not need to be capable of expressing an exogenous nucleic acid to be useful; such cells could be used for expanding the nucleic acid. The use of such host cells for such purposes was broadly known to molecular biologists in November 1992.

11. Other host cells are described in the patent application identified at page 1, lines 24-28, which is now U.S. Patent No. 5,648,267. I understand that this patent, of which I am the inventor, forms a part of the application identified above. The patent describes, for example, an *E. coli* host cell used to expand a cloned nucleic acid (rather than express the encoded protein) in the paragraph from column 1, line 59 to column 2, line 7.

12. I have been asked to comment on whether the application describes a host cell that does not necessarily secrete an antibody protein. In my opinion, the application would have

conveyed this concept clearly to a person skilled in the field in November 1992. First, as I explain above, the application describes host cells that do not necessarily express exogenous nucleic acid sequences, such as host cells used for expanding nucleic acids. If a host cell did not express a nucleic acid sequence, it would not secrete the polypeptide it encodes. Second, at page 19, lines 22-29, the application states that a chimeric antibody may be produced through *in vitro* assembly of separately expressed immunoglobulin chains. A person skilled in this field in November 1992 would have recognized that such a technique would not depend on the use of cells that secreted the immunoglobulin chains. For at least these reasons, I believe that the application would have informed a person of ordinary skill that the capability to secrete antibody proteins was not an essential or necessary feature of host cells such as those having the characteristics identified in paragraph 3 above.

13. I have been asked to comment on the observation in the final Office action that the claims "encompass host cells that express nucleic acids encoding membrane-bound anti-CD20 antibody." I note that none of the claims requires or makes reference to a membrane-bound antibody.

14. "Antibodies" are broadly described in the application, for example, at page 11, line 16 to page 12, line 2. The structural and functional characteristics of the different classes of antibodies were well known in the art by November 1992. *See*, *e.g.*, the textbook by D. Male *et al.*, *Advanced Immunology*, 2d. ed. (Philadelphia: J.B. Lippincott Co.), 1991, pages 2.1-2.7, a copy of which is attached as an exhibit to this declaration. It was further known that antibodies occur in both soluble and membrane-bound forms. *See id.*, *e.g.*, at page 2.2, paragraph bridging columns. I believe that a person skilled in the art in November 1992 would have understood the use of the general term "antibody," as it appears in our application, to be consistent with its conventional usage in the art, and in particular, to refer to both soluble and membrane-bound forms of antibodies.

15. In summary, it is my opinion that the application fully describes the nature of the host cells that would be suitable for the various purposes discussed in the application, according to conventional practice in the field. By November 1992, the level of skill in molecular biology was high, and it was not necessary to provide minute detail to describe

every host cell or method for introducing nucleic acids into a host cell in a technical communication.

16. One skilled in this field in November 1992 would have understood that the components and attributes of various host cells are determined according to the particular functions that such host cells need to perform. A person skilled in the field would have understood that the presence of the necessary components and attributes for achieving a stated purpose is implicit in a reference to a particular cell as a "host cell" for that purpose. It is my opinion that the application identifies and sufficiently describes host cells having all of the molecular components necessary for replicating nucleic acids encoding immunoglobulin heavy chains and light chains and, as necessary, for producing or secreting antibody proteins.

17. Taking into account all of the different kinds of host cells described in our application, it is my opinion that the application fully describes the subject matter in paragraph 3 above for a person skilled in the art of molecular biology in November 1992. In particular, the application describes host cells comprising nucleic acid introduced by one or more plasmids, or by other means; host cells that are used to produce antibody proteins, and those that are not; as well as host cells that are capable of secreting antibody proteins, and those that need not secrete antibody proteins. I believe the application would have conveyed to a person skilled in molecular biology in November 1992 that all such host cells are useful in methods that were well known in the art for expanding, or expressing nucleic acid sequences encoding immunologically active anti-CD20 antibodies according to the procedures described in the patent application.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent subject to this reexamination proceeding.

Mitchell E. Reff

06/08/2007

Date

EXHIBIT A

CURRICULUM VITAE

MITCHELL ELLIOT REFF

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PLACE OF BIRTH:	May 6, 1951 Queens, New York
EDUCATION:	1973 - B.S. Chemistry Massachusetts Institute of Technology 1976 - Ph.D. Medical Microbiology Stanford Medical School
THESIS:	Phylogenetic Relationships of Prokaryotes Based on Characterization on their Ribosomal RNA
MARITAL STATUS:	Married in 1977, two adult children

RESEARCH AND PROFESSIONAL EXPERIENCE:

04/12/06-present	**Biogen Idec Inc,** San Diego, CA Vice President, Discovery Oncology Manage multiple bicoastal oncology groups developing small molecule and recombinant protein therapeutic molecules for oncology
01/01/04-04/12/06	**Biogen Idec Inc,** San Diego, CA Vice President, Molecular Engineering Manage a new research department, which develops novel protein molecules with altered properties to enhance pharmaceutical utility. Manage a research department, which develops mammalian production cell lines secreting monoclonal antibodies and other recombinant proteins for clinical trials and improves manufacturing capability of host cells. Both of the above bicoastal responsibilities. Manage several oncology groups developing small molecule and recombinant protein therapeutic molecules for oncology.
11/03-12/31/03	**Biogen Idec Inc**, San Diego CA Vice President, Molecular Biology and Antibody Discovery Manage a research department, which develops mammalian production cell lines secreting monoclonal antibodies and other recombinant proteins for clinical trials. In addition, all of the responsibilities below under the next three subheadings
01/02–05/04	Project Leader for Cancer Target Discovery Program discovering and validating novel tumor specific proteins useful as targets for monoclonal antibody therapy of solid tumors.
10/16/02-11/03	**Idec Pharmaceuticals Corporation**, San Diego, CA Vice President, Molecular Biology and Antibody Discovery Manage a department, which conducts feasibility studies and establishes collaborations that could potentially lead to the generation of therapeutic antibodies to treat autoimmune diseases and malignancies. Manage a department, which generates and characterizes monoclonal antibodies.
10/95 – 10/15/02	**Idec Pharmaceuticals Corporation**, San Diego, CA Senior Director of Molecular Biology Manage a department, which discovers and expresses novel proteins useful as targets for cancer vaccines or anti-tumor monoclonal antibodies. Manage a department, which applies protein-engineering methodology to improve the properties of antibody molecules and enhance IDEC's proprietary position in antibody technology.

Manage a department, which clones and expresses recombinant proteins or constructs cells lines containing recombinant proteins as substrates for functional assays.
Manage a department, which develops mammalian production cell lines secreting monoclonal antibodies and other recombinant proteins for clinical trials.
Involved in academic collaborations and strategic alliances to investigate metabolic engineering of mammalian cell lines for enhanced productivity.
Involved in strategic alliances studying alternative manufacturing sources of complex recombinant proteins.
Involved in strategic alliances utilizing mammalian expression technology developed at IDEC Pharmaceuticals.
Involved in academic collaborations and strategic alliances to understand the mechanism of action of IDEC's anti-CD23 antibody and potentially evaluate molecules that may be therapeutic in allergic asthma and allergic rhinitis.
Involved in academic collaborations of protein engineering to improve the properties of antibody molecules.

10/95-10/00 **Idec Pharmaceuticals Corporation**, San Diego, CA
Project Leader targeting CD23 antigen (allergic asthma, allergic rhinitis, chronic lymphocytic leukemia) using a chimeric primate-human (PRIMATIZED®) monoclonal antibody. Led project during pre-clinical development through successful IND submission and Phase I clinical trials.

06/01/94-10/95 **Idec Pharmaceuticals Corporation**, San Diego, CA
Senior Director of Gene Expression
Involved in strategic alliances utilizing mammalian expression technology developed at IDEC Pharmaceuticals.
Manage a department, which develops mammalian production cell lines secreting monoclonal antibodies and other recombinant proteins for clinical trials.

05/21/90-05/31/94 **Idec Pharmaceuticals Corporation**, San Diego, CA
Director of Gene Expression
Project Leader targeting B cell lymphoma using a chimeric mouse-human anti-CD20 monoclonal antibody (Rituxan). Rituxan was approved in the U.S. for Non-Hodgkin's B-cell Lymphoma in 1997. Project Leader from pre-clinical development until Rituxan entered pivotal clinical trial.
Developed the mammalian cell line used to manufacture Rituxan.
Manage a research program and perform research to develop monoclonal antibodies for clinical trials.

03/85-05/90	**Smithkline & French Laboratories**, Philadelphia, PA Assistant Director Department of Gene Expression Sciences Management of a research program to develop novel thrombolytic compounds. Development of high-level gene expression in novel mammalian cell lines useful for large-scale production.
10/83-05/90	**Temple University School Of Medicine**, Philadelphia, PA Adjunct Assistant Professor Department of Microbiology & Immunology Perform basic genetic research involving both transient and stable expression of bacterial genes in mammalian cells as a mechanism for understanding the regulation of gene expression in eukaryotes. Graduate student received Ph.D. while studying the effects of alterations in DNA sequences on the accuracy and efficiency of polyadenylation.
09/82-03/85	**Smithkline & French Laboratories**, Philadelphia, PA Senior Investigator Molecular Genetics Department Perform basic genetic research involving the expression of genes in mammalian cells with the goal of developing expression vectors to maximize production of given pharmaceutical products.

09/81-08/82 **National Cancer Institute,** Bethesda, MD
Senior Staff Fellow
Cellular Regulation Section, Laboratory of Biochemistry
Perform basic genetic research involving transient expression of bacterial genes in mammalian cells as a mechanism for understanding the regulation of gene expression in eukaryotes.

07/80-09/81 **National Institute On Aging**, NIH, Bethesda, MD
Special Assistant to the Associate Director
Performed administrative duties including contract review, strategic policy development, conference evaluation, and budget review. Assisted in the development of a five-year National Research Plan on Aging. Planned, convened, and co-chaired conference and editor of a book on biological markers of aging. Served as an acting health science administrator in the area of molecular biology.

09/79-08/81 **University Of Colorado Health Sciences Center**, Denver, CO
Assistant Professor
Department of Biochemistry, Biophysics and Genetics
Staff scientist at the Davis Institute (see below).
Intergovernmental Personnel Agreement position at the National Institute on Aging (see above).

09/79-06/80 **Davis Institute On Aging**, Denver, CO
Staff Scientist
Designed and equipped new laboratory. Initiated research in areas of cell biology and biochemistry.

10/76-06/79 **Harvard Medical School**
Postdoctoral Research Fellow
Department of Microbiology and Molecular Genetics and
CHILDREN'S HOSPITAL MEDICAL CENTER, Boston, MA
Department of Clinical Genetics
Carried out independent research in genetics.

HONORS:

1974-1976	Public Health Service Traineeship
1981	Public Health Service Award for Outstanding Performance as Special Assistant to the Associate Director for Biomedical Research and Clinical Medicine
1997	Excellence Award Once-a-year award chosen by the Executive Committee of IDEC Pharmaceuticals

REFERENCES:

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GRANTS:

CTL Induction by Soluble HPV 16 E7 Antigen
Co-Principal Investigator: Effort on Project 5%
Grant # 1 R43 AI36778-01A1
August 1995 to January 1996

Broadly RSV Neutralizing Human Monoclonal Antibodies
Co-Principal Investigator: Effort on Project 12%
Grant # 1 R43 AI36027-01
October 1995 to September 1997

Humanized Anti-gp39 Antibody for Antoimmune/Graft Rejection
Senior Director: Effort on Project 10%
Grant # 1 R43 AI39326-02
September 1997 to September 1999

PATENTS: REFF, M.E.

1. Expression Vector. Filed, U.S. Patent Office, 1985. SKB 14260. Abandoned.
2. Novel Cell Line for Production. Filed, U.S. Patent Office, 1986. SKB 14317. Abandoned.
3. Novel System for High Level Expression in Mammalian Cells.Filed, U.S. Patent Office, 1987. SKB 14391. Abandoned.
4. Novel Fibrinolytics. Filed, U.S. Patent Office, 1988. SKB 14403 and SKB 14404. Abandoned.
5. Therapeutic Application of Chimeric and Radiolabeled Antibodies to Human B Lymphocyte Restricted Differentiation Antigen for Treatment of B Cell Lymphoma.
   Filed, U.S. Patent Office, November 3, 1993 - **US Patent 5,736,137**, April 7, 1998.
   Filed, U.S. Patent Office, June 7, 1995 - **US Patent 5,776,456**, July 7, 1998.
   Filed, U.S. Patent Office, June 7, 1995 - **US Patent 5,843,439**, December 1, 1998.
   Filed, U.S. Patent Office, June 7, 1995 - **US Patent 6,399,061,** June 4, 2002.
   Filed, U.S. Patent Office, June 7, 1995 – **US Patent 6,682,734,** Jan 27,2004.
   Filed, U.S. Patent Office, August 29, 1997 - US 08/921,060, Pending.
6. Impaired Dominant Selectable Marker Sequence and Intronic Insertion Strategies for Enhancement of Expression of Gene Product and Expression Vector Systems Comprising Same.
   Filed, U.S. Patent Office, November 3, 1993 - **US Patent 5,648,267**, July 15, 1997.
   Filed, U.S. Patent Office, June 7, 1995 - **US Patent 5,733,779**, March 31, 1998.
   Filed, U.S. Patent Office, Jan 26, 1998 - **US Patent 6,017,733** January 25,2000
   Filed, U.S. Patent Office, Mar 30,1999 – **US Patent 6,159,730** December 12, 2000
   Filed, U.S. Patent Office, July 13, 2000 – US 09/615,796 Pending
7. Recombinant Anti-CD4 Antibodies for Human Therapy.
   Filed, U.S. Patent Office, September 6, 1995 - **US Patent 6,136,310** October 24, 2000.
8. Gamma-1 and Gamma 3 Anti-Human CD23 Monoclonal Antibodies and Use Thereof as Therapeutics.
   Filed, U.S. Patent Office, February 20, 1997 - **US Patent 6,011,138** Jan 04, 2000.
   Filed, U.S. Patent Office, February 5, 1998 – **US Patent 6,893,636** May 17, 2005.
9. Method for Integrating Genes at Specific Sites in Mammalian Cells via Homologous Recombination and Vectors for Accomplishing the Same
   Filed, U.S. Patent Office, March 14, 1997 - **U.S. Patent 5,830,698**, November 5, 1998.
   Filed, U.S. Patent Office, February 13, 1998 – **U.S. Patent 5,998,144** December 7,1999
   Filed, U.S. Patent Office, June 30,1999 - **US Patent 6,413,777** July 2,2002
   Filed, U.S. Paten Office, April 1, 2002-**US Patent 6,841,383** January 11,2005
10. Variant IgG3 Rituxan and Therapeutic use thereof.
    Filed, U.S. Patent Office, October 10,2001 09/982849
11. Recombinant Antibodies Co-expressed with GNT III
    Filed U.S. Patent Office, April 2, 2002 10/113,929
12. Inhibition of Apoptosis Process and Improvement of Cell Performance
    Filed US Patent Office, July 10, 2002 10/191,052
13. Polycistronic Expression of Antibodies
    Filed U.S. Patent Office, August 5, 2002 60/400,687
14. Modified Binding Molecules Comprising Connecting Peptides
    Filed U.S Patent Office, June 27,2003 60/483,877

PUBLICATIONS:

1. Reff, M.E. and Stanbridge, E.J.: Conformational differences in bacterial ribosomal RNAs under nondenaturing conditions. Nature 260:724-726, 1976.

2. Reff, M.E., Stanbridge, E.J., and Schneider, E.L.: Phylogenetic relationships between mycoplasmas and other prokaryotes based upon the electrophoretic behavior of their ribosomal RNAs. Int. J. System Bact. 27:185-193, 1977.

3. Reff, M.E. and Davidson, R.L.: *In vitro* DNA dependent synthesis of globin RNA sequences from erythroleukemic cell chromatin. Nucl. Acids Res. 6:276-287, 1979.

4. Reff, M.E. and Davidson, R.L.: Deoxycytidine reverses the suppression of pigmentation caused by 5-BrdUrd without changing the distribution of 5-BrdUrd in the DNA. J. Biol. Chem. 254:6869-6872, 1979.

5. Schneider, E.L. and Reff, M.E.: Cell Culture Aging. Mol. Cell. Bio. 36:169-176, 1981.

6. Kato, H., Reff, M., and Schneider, E.L.: Analysis of regenerating hepatic cell replication *in vivo.* Cell and Tissue Kinetics 15:637-642, 1982.

7. Pfarr, D., Sathe, G., and Reff, M.E.: A highly modular cloning vector for the analysis of eukaryotic genes and gene regulatory elements. DNA 4:461-467, 1985.

8. Pfarr, D., Rieser, L., Woychik, R., Rottman, M., and Reff, M.E.: Differential effects of polyadenylation regions on gene expression in mammalian cells. DNA 5:115-122, 1986.

9. Gimmi, E.R., Soprano, K.J., Rosenberg, M., and Reff, M.E.: Deletions in the SV40 late polyadenylation region downstream of the AAUAAA show similar effects on expression in various mammalian cell lines. Nucl. Acids Res. 16:8977-8997, 1988.

10. Connors, R.W., Noverale, J., Sweet, R.W., Berkowitz, B.B., Williams, D., Franklin, S., Shebuski, R., Trill, J.J., Pfarr, D., and Reff, M.E.: DHFR coamplification of tissue plasminogen activator in DHFR positive bovine endothelial cells: *In vitro* characterization of the purified serine protease. DNA 7:651-661, 1988.

11. Lijnen, H.R., Pierard, L., Reff, M.E., and Gheysen, D.: Characterization of a chimaeric plasminogen activator obtained by insertion of the second kringle structure of tissue-type plasminogen activator (amino acids 173 through 262) between residues ASP^{130} and SER^{139} of Urokinase-type plasminogen activator. Thrombosis Research 52:431-441, 1988.

12. Pierard, L., Quintana, L.G., Reff, M.E., and Bollen, A.: Production in eukaryotic cells and characterization of four hybrids of tissue-type and urokinase type plasminogen activators. DNA 8:321-328, 1989.

13. Gimmi, E.R., Reff, M.E., and Deckman, I.C.: Alterations in the pre-mRNA topology of the bovine growth hormone polyadenylation region decrease poly(A) site efficiency. Nucl. Acids Res. 17:6983-6998, 1989.

14. Trill, J.J., Fong, K-L., Shebuski, R.J., McDevitt, P., Rosa, M.D., Johanson, K., Williams, D., Boyle, K.E., Sellers, T.S., and Reff, M.E.: Expression and characterization of finger protease (FP); a mutant tissue-type plasminogen activator (t-PA) with improved pharmacokinetics. Fibrinolysis 4:131-140, 1990.

15. Gennaro, D.E., Hoffstein, S.T., Marks, G., Ramos, L., Oka, M.S., Reff, M.E., Hart, T.K., and Bugelski, P.J.: Quantitative immunocytochemical staining for recombinant tissue-type plasminogen activator in transfected Chinese hamster ovary cells. Proc. Soc. Exp. Biol. Med. 198:591-598, 1991.

16. Newman, R., Alberts, J., Anderson, D., Carner, K., Heard, C., Norton, F., Raab, R., Reff, M., Shuey, S., and Hanna, N.: "Primatization" of recombinant antibodies for immunotherapy of human diseases: A macaque/human chimeric antibody against human CD4. Bio/Technology 10:1455-1460, 1992.

17. Reff, M.E.: High-level production of recombinant immunoglobulins in mammalian cells. Current Opinion in Biotechnology 4:573-576, 1993.

18. Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.C., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N., and Anderson, D.R.: Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 83:435-445, 1994.

19. Okafo, G.N., Burrow, L.M., Neville, W., Truneh, A., Smith, R.A.G., Reff, M.E., and Camilleri, P.: Simple differentiation between core-fucosylated and nonfucosylated gycans by capillary electrophoresis. Analytical Biochemistry 240:68-94, 1996.

20. Anderson, D., Chambers, K., Hanna, N., Leonard, J., Reff, M., Newman, R., Baldoni, J., Dunleavy, D., Reddy, M., Sweet, R., and Truneh, A.: A Primatized MAb to Human CD4 Causes Receptor Modulation, without Marked Reduction in $CD4^+$ T Cells in Chimpanzees: *In Vitro* and *In Vivo* Characterization of a MAb (IDEC-CE9.1) to Human CD4. Clinical Immunology and Immunopathology 84:73-84, 1997.

21. Preston, M.J., Gerceker, A.A., Reff, M. Production and Characterization of a Set of Mouse-Human Chimeric Immunoglobulin-G (IgG) Subclass and IgA Monoclonal-Antibodies with Identical Variable Regions Specific for Pseudomonas-Aeruginosa Serogroup 06 Lipopolysaccharide. Infect. Immun. 66:4137-4142, 1998.

22. Heard, C., Brams, P., Walsh, E., Huynh, T., Chamat, S., Reff, ME, Owyang, A., Shestowsky, W., and Newman, R. Two Neutralizing Human Anti-RSV Antibodies: Cloning Expression and Characterization. Molecular Medicine 5:35-45, 1999.

23. Yron, I., Deckert, M., Reff, ME., Schwartz, MA. and Altman, A. Integrin-dependent Tyrosine Phosphorylation and Growth Regulation by Vav. Cell Adhesion & Communication 7:1-11, 1999.

24. Nakamura, T., Kloetzer, W.S., Brams, P., Hariharan, K., Chamat, S., Cao, X., LaBarre, M.J., Chinn, P.C., Morena, R.A, Shestowsky, W.S., Li, Y-P., Chen, A., and Reff, M.E. *In Vitro* IgE inhibition in B cells by anti-CD23 monoclonal antibodies is functionally dependent on the immunoglobulin Fc domain. International Journal of Immunopharmocology, 22:131-141, 2000

25. Reddy, M.P., Kinney, C.A.S., Chaikin, M.A., Payne, A., Fishman-Lobell, J., Tsui, P., Dal Monte, P.R., Doyle, M.L., Brigham-Burke, M.R., Anderson, D., Reff, M., Newman, R., Hanna, N., Sweet, R.W., and Truneh, A. Elimination of Fc receptor-dependent effector functions of a modified IgG4 mAb to human CD4. Journal of Immunology, 164:1925-1933, 2000

26. Newman, Roland, Hariharan, Kandasamy, Reff, Mitchell, Anderson, Darrell R., Braslawsky, Gary, Santoro, Denise, Hanna, Nabil, Bugelski, Peter J., Brigham-Burke, Michael, Crysler, Carl, Ganon, Robert C., Dal Monte, Paul, Doyle, Michael L., Hensley, Preston, Reddy, Manjula P., Swet, Raymond W., and Alemseged Truneh. Modification of the Fc Region of a Primatized IgG Antibody to Human CD4 Retains Its Ability to Modulate CD4 Receptors but Does Not Deplete CD4+ T Cells in Chimpanzees. Clinical Immunology, 98:164-174, 2001

27. Davies, Julian and Reff, Mitchell. Chromosome localization and gene copy number quantitation of three random integrations in CHO cells and their amplified cell lines using fluorescent *in-situ* hybridization. Biotechnology and Applied Biochemistry 33 (part 2): 99-105, 2001

28. Davies, Julian, Jiang LiYing, LaBarre, Michael J, Anderson Darrell, and Reff, Mitchell. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FCgammaRIII. Biotechnology and Bioengineering. 74:288-94, 2001.

29. Reff, Mitchell E, and Heard, Cheryl. A review of modifications to recombinant antibodies: attempt to increase efficacy in oncology applications. Critical Reviews in Oncology/ Hematology. 40:25-35, 2001

30. Reff, M., Braslawsky, G. and Hanna, N. Future Approaches for Treating Hematologic Disease Current Pharmaceutical Biotechnology 2:369-382, 2001

31. Yabuuchi, Shingo, Nakamura, Takehiko, Kloetzer, William S. and Reff, Mitchell E. Anti-CD23 monoclonal antibody inhibits germline C transcription in B cells. International Immunopharmacology, 2:453-461, 2002

32. Reff, M., Hariharan, K. and Braslawsky, G. Future of Monoclonal Antibodies in the Treatment of Hematologic Malignancies. Cancer Control. 9:152-166, 2002

33. Poole, Jill A., Meng, Jianfeng, Reff, Mitchell, Spellman, Mary C. and Rosenwasser, Lanny J.; Anti-CD23 monoclonal antibody , lumiliximab, inhibited allergen-induced responses in antigen-presenting cells and T cells from atopic subjects. Journal of Allergy and Clinical Immunology, 116:780-788, 2005

34. Glaser, Scott M., Hughes, Ina E., Hopp, Jennifer R., Hathaway, Karen, Perret, Daniel and Reff, Mitchell E., Novel Antibody Hinge Regions for Efficient Production of CH2 Domain-deleted antibodies. Journal of Biological Chemistry, 280:41494-41503, 2005

35. Lukashev, Matvey, LePage, Doreen, Wilson, Cheryl, Bailly, Veronique, Garber, Ellen, Lukashin, Alex, Ngam-ek, Apinya, Zeng, Weike, Allaire, Norman, Perrin, Steve, Xu, Xianghong, Szeliga, Kendall, Wortham, Kathleen, Kelly, Rebecca, Bottiglio, Cindy, Ding, Jane,Griffith, Linsa Heaney, Glenna, Silverio, Erika, Yang, William, Jarpe, Matt, Fawell, Steve, Reff, Mitchell, Carmillo, Amie, Meier, Werner, Violette, Shelia M., Mackay, Favienne, Yang Dajun, Hoffman, Robert, and Browning, Jeffrey L., Targeting the Lymphotoxin-β Receptor with Agonist Antibodies as a Potential Cancer Therapy. Cancer Research, 66:9617-9624, 2006

36 Figueroa, B. Jr, Ailor, E., Osborne, D., Hardwick, J.M., Reff, M. and Betenbaugh, M.J., Enhanced cell culture performance using inducible anti-apoptotic genes E1B-19K and Aven in the production of a monoclonal antibody with Chinese hamster ovary cells. Biotechnology Bioengineering, Epub Nov 10, 2006

BOOKS, BOOK CHAPTERS, OTHER PUBLICATIONS

1. Stanbridge, E.J. and Reff, M.E.: The molecular biology of the mycoplasmas. In: The Mycoplasmas, M.F. Barile and S. Razin (eds.), Vol. 1, Cell Biology pp. 157-185, Academic Press, 1979.

2. Reff, M.E. and Schneider, E.L.: (eds.) Biological Markers of Aging Conference, U.S. Department of Health and Human Services Publication, NIH Publication No. 82-2221, April 1982.

3. Reff, M.E.: Aging at the molecular level. Invited position paper for the United States Office of Technology Assessment, Washington, D.C. 20510, 1983.

4. David, I., Fabricant, J.D., Gerhard, J., Letvin, J., Mukherjee, A., Reff, M.E., and Reppert, S.: Developmental Biology, Genetics and Aging. In: Life Sciences Experiments for a Space Station. J. Fabricant (ed.) pp. 20-30. University of Texas Medical Branch, Galveston, TX 1983.

5. Johansen, H., Reff, M.E., Schumperli, D., and Rosenberg, M.: Using the *E. coli* galactokinase gene to study gene regulatory elements. In: Gene Expression: The Translational Step and its Control (Alfred Benson Symposium 19) B.F.C. Clark and H.U. Peterson (eds.) Munskaard Copenhagen, 1984.

6. Reff, M.E.: RNA and protein metabolism. In: Handbook of the Biology of Aging (2nd Edition) C.E. Finch and E.L. Schneider (eds.) Van Nostrand Reinhold, pp. 225-254, 1985.

7. Rosenberg, M., Brawner, M., Gorman, J., and Reff, M.E.: Using galactokinase gene fusion to study gene regulation in E. coli, streptomyces, yeast, and higher cell systems. In: Genetic Engineering, Volume 8, J.K. Setlow (ed.), Plenum Press, pp. 151-180, 1986.

8. Reff, M.E. and Pfarr, D.S.: Production of proteins by mammalian cells using dominant selectable and amplifiable markers. In: Gene Amplification in Mammalian Cells R.E. Kellems (ed.), Marcel Dekker, Inc. pp. 355-367, 1992.

9. Barnett, R.S., Limoli, K.L., Huynh, T.B., Ople, E.A., and Reff, M.E.: Antibody Production in Chinese Hamster Ovary Cells Using an Impaired Selectable Marker. In: Antibody Expression and Engineering H.Y. Wang and T. Imanaka (eds.), (ACS Symposium Series 604). pp 27-40, 1995.

10. Reff, M.: Homologous Recombination in CHO Cells. BioPharm 11: 33-34, 1998.

11. Doyle, M., Brigham-Burke, M., Blackburn, M.N., Brooks, I.S., Smith, T.M., Newman, R., Reff, M.E., Stafford, III, W.F., Sweet, R.M., Truneh, A., Hensley, P., and O'Shannessy, D.J. Measurement of Protein Interaction Bioenergetics: Application to Structural Variants of an Anti-CD4 Antibody. In Energetic of Biological Macromolecules, Part C Methods in Enzymology M.L. Johnson and G.K. Ackersn (eds), 323: 207-230, 2000.

12. Reff, M. Efficacy of the anti-angiogenic approach to solid tumors. Letter in Drug Discovery Today, 6,716-717,2001

13. Reff, M. The Discovery of Rituxan. In The Process of New Drug Discovery and Development Second Edition C.G. Smith and J. T. O'Donnell (eds.), Informa Healthcare USA, Inc. pp.565-584,2006

ABSTRACTS:

1. Reff, M.E., Stanbridge, E.J., and Schneider, E.L.: Electrophoretic migratory differences in the ribosomal RNA of bacteria, L forms acholeplasma and mycoplasma. Abst. of the Annual Meeting of the American Society for Microbiology, 1975, 59.

2. Johansen, H., Ferguson, B., Berman, R., Reff, M., and Rosenberg, M.: Translation regulation in mammalian cells studied by gene fusion. DNA 3:112, 1984.
Fourth Annual Congress for Recombinant DNA Research, 1994

3. Trill, J., Pfarr, D., and Reff, M.E.: Efficiency of transfection of linear DNA with and without pBR sequences. Fed Proc. 46(6) 1987 2040. 78th Annual Meeting of the American Society of Biological Chemists, Philadelphia, PA June 7-11, 1987.

4. Connors, R., Sweet, R., Pfarr, D., and Reff, M.E.: Novel mammalian overexpression systems. 2nd Annual American Society of Microbiology Conference in Biotechnology, 1987.

5. Gimmi, E.R., Deckman, I.C., and Reff, M.E.: The role of pre-mRNA secondary structure in mammalian polyadenylation. Journal of Cellular Biochemistry, Supplement 0 (12 Part D), 1988. 40 Symposia on Molecular Biology of RNA, UCLA Symposia on Molecular & Cellular Biology, Keystone, CO April 4-10, 1998.

6. Connors, R.W., Trill, J., Sweet, R., Shebuski R., and Reff, M.E.: Overexpression of tPA in Bovine Endothelial cells and characterization of the protein product. FASEB Journal 2,(4) 1988 A561, 72nd annual meeting of the Federation of American Societies for Experimental Biology, Las ,Vegas NV May 1-5, 1988.

7. Connors, R.W., Noverale, J., Sweet, R.W., Berkowitz, B.B., Williams, D., Franklin, S., Shebuski, R., Trill, J.J., Pfarr, D., and Reff, M.E.: DHFR coamplification of tissue plasminogen activator in DHFR positive bovine endothelial cells: *In vitro* characterization of the purified serine protease. Ninth International Congress on Fibrinolysis, Brussels. Belgium 1988.

8. Fong, L.L.L., Cotter, L.M., Fujita, A., Chen, T.K., Reff, M.E., Crysler, C.S. Boyle, K.E., Pfarr, D.S., Trill, J.J., Connors, R., Noveral, J., Williams, D., Berkowitz, B.A., Mico, B.A., and Shebuski, R.: Comparative pharmacokinetics of recombinant tissue-type plasminogen activator (tPA) derived from four different cell liens. Ninth International Congress on Fibrinolysis Brussels. Belgium 1988.

9. Pfarr, D., Trill, J., and Reff, M.: DHFR coamplification of tPA using a tripartite expression vector. J.Cell Biol 107 (6 part3) 1988 326A. American Society Cell Biology /American Society for Biochemisty and Molecular Biology Joint meeting, San Francisco, CA January 29th-February 2nd 1989.

10. Reff, M., Trill, J., Fujita, T., Fong, K.L., Cotter, L., Storer, B., and Shebuski, R.J.: Pharmacological efficacy and pharmacokinetics of a finger-protease (FP) tPA mutant in a rabbit model of peripheral arterial thrombosis. J.Cell Biol 107 (6 part3) 1988 617A. American Society Cell Biology /American Society for Biochemisty and Molecular Biology Joint meeting, San Francisco, CA January 29th-February 2nd

11. Trill, J., Pfarr, D., Sathe, G., and Reff, M.E.: High efficiency of expression of tissue plasminogen activator through the use of a polycistronic plasmid. J.Cell Biol 107 (6 part3) 1988 326A. American Society Cell Biology /American Society for Biochemisty and Molecular Biology Joint meeting, San Francisco, CA January 29th-February 2nd 1989.

12. Gimmi, E.R., Reff, M.E., and Deckman, I.C.: Alterations in the pre-messenger RNA topology of the bovine growth hormone polyadenylation region affect polyadenylation site efficiency. RNA Processing, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pg. 89 1989.

13. Pierard, L., Quintana, G., Reff, M.E., Bollen, A., Secretion of recombinant plasminogen activators by mouse myeloma cells.. Meded Fac Landbouwwet Rijksuniv Gent 54(4B) 1989 1569-1572. Third Forum for Applied Biotechnology, September 28th, 1989

14. Trill, J.J., McLeod, K., Fong, K.L., Marks, G.J., Bugelski, P.J., Reff, M.E., Devine, J.M., Edwards, R.M., Fallon, A., Craig, S., Hughes, P., and Dawson, K.M.: Site specific mutations in the growth factor domain of tissue-type plasminogen activator (tPA) reduce hepatic clearance. Fibrinolysis 4 Suppl 3 1990 109 Tenth International Congress on Fibrinolysis, Indianapolis, IN. August 4-8 1990.

15. Reff, M.E.: Splicing and gene expression of heterologous genes in mammalian cells.).Abstr Pap Am Chem Soc 199(1-2)1990 Biot 129. 19th American Chemical Society National Meeting & Exposition Boston, MA April 22-27, 1990

16. Reff, M.E., Anderson, D. R., Rabb, R. Carner, K. Alberts, J., and Newman, R.: Cloning and expression of a chimeric anti-CD20 monoclonal antibody. Proceedings of the 2nd Annual IBC International Conference on Antibody Engineering; San Diego CA. December 16-18, 1991

17. Anderson, D.R., McCoobery, R.E., Reff, M.E., Newman, R., Raychaudhuri, S., and Hanna, N.: Immunoreactivity and effector function associated with a chimeric anti-CD20 antibody. Proceedings of the 2nd Annual IBC International Conference on Antibody Engineering; 1991. December 16-18, San Diego CA.

18. Newman, R., Alberts, J., Anderson, D., Norton, F., Raab, R., Reff, M., and Hanna, N.: Primatized antibodies for immunotherapy: A of human/macaque chimeric antibody against human CD4. Second International Conference on Human Antibodies and Hybridomas, Cambridge, UK ,March 24-26, 1992.

19. Anderson, D., Reff, M., Chambers, K., Newman, R., Hanna, N., and Leonard, J.: Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. Blood 1992, November 80(10): 191a. The American Society of Hematology, 34th Annual Meeting (1992).

20. Reff, M.E., Leonard, J., Newman, R., Hanna, N., and Anderson, D.: Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Journal of Cellular Biochemistry; Suppl 17E, pp260, 1993. Keystone Symposia on Molecular & Cellular Biology ,April 17-25, 1993.

21. Grillo-López, A.J., Anderson, D.R., Leonard, J.E., Reff, M.E., Caralli, V., and Dallaire, B.K. Monoclonal anti-CD20 antibody (IDEC-C2B8) therapy of B-cell non-hodgkins lymphoma - preclinical development and early clinical results. Antibody Immunoconjugates and Radiopharmaceuticals 1994; 7 (1), 91. Ninth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, March, 1994..

22. Grillo-López, A.J., Caralli, V., Leonard, J.E., Dallaire, B.K., Anderson, D.R., and Reff, M.E.: Monoclonal anti-CD20 antibody (IDEC-C2B8) therapy of B-cell non-Hodgkin's lymphoma - pre clinical development and early clinical results. Proceedings of the Eighth NCI/EORTC Symposium on New Drugs in Cancer Therapy; Amsterdam, March 15-18, 1994.

23. Reff, M.E.: High level expression of MAbs in CHO cells. Journal of Cellular Biochemistry Supplement o (18D) p188, 1994. Keystone Symposium on Antibody Engineering: Research and Application of Genes Encoding Immunoglobulins, Lake Tahoe, CA March 7-13, 1994.

24. Reff, M.E.: High level expression of MAbs in CHO cells. Abstracts of Papers American Chemical Society 207(1-2) p BIOT 54, 1994. 207th National Meeting of the American Chemical Society, San Diego, CA March 13-17, 1994.

25. Grillo-López, A.J., Leonard, J.E., Caralli, V., Reff, M.E., Anderson, D.R., and Dallaire, B.K.: IDEC-C2B8. A promising new monoclonal anti-CD20 antibody for the treatment of B-cell non-Hodgkin's lymphoma. La Revista de Investigacion Clinica 1994, Abril: 204 International Society of Hematology Meeting, April 1994..

26. Reff, M.E., Adams K., and Anderson, D.R.: Comparison of recombinant chimeric immunoglobulin secreted and purified from Chinese Hamster Ovary (CHO) and murine SP2/0 myeloma cells. American Chemical Society International Conference "Recovery of Biological Products VII", September 25-30, 1994, San Diego, CA.

27. Reff, M.E.: High level protein production in CHO cells using an impaired selectable marker. Journal of the Society for In Vitro Biology 32 (3 Part II), 1996. World Congress on In Vitro Biology, June 22-27, 1996. San Francisco.

28. Nakamura, Takehiko; Brams, Peter; Cao, Xianjun; Chamat, Soulaima;Chen, Agnes; Chinn, Paul; Hanna, Nabil; Hariharan, Kandasamy; Kloetzer, William; LaBarre, Michael; Li, Yan-Ping; Morena, Ron; Reff, Mitchell; Shestowsky, William In vitro suppression of IgE synthesis by a primatized monoclonal antibody (mab) against human CD23 antigen. 1999 FASEB 3 No 5 PART 2 A989.

29. Reff, Mitchell; Pathan, Nuzhat; Hariharan, Kandasamy; Hanna, Nabil;Grint, Paul; Hopkins, Michael; Saven, Alan. Induction of apoptosis by IDEC-152 (anti-CD23) in lymphoma cells. Blood VOL. 98 NO. 11 Part 1, 2001 367a. 43rd Annual Meeting of the American Society of Hematology, Part 1 December 7-11, 2001.

30. Pathan, Nuzhat; Berquist, Lisa; Grint, Paul; Hanna, Nabil; Hariharan,Kandasamy; Hopkins, Michael; Reff, Mitchell; Saven, Alan. Induction of apoptosis by IDEC-152 (anti-CD23) in chronic lymphocytic leukemia. Proceedings of the American Association for Cancer Research Annual Meeting VOL. 43 March, 2002 PP. 1005. 93rd Annual Meeting of the American Association for Cancer Research. April 6-10, 2002

31. Reff, Mitchell E. : Am amto-CD23 antibody which blocks IgE production in the treatment of allergic asthma. IBC 13th International Conference Antibody Engineering Dec 2-4, 2002, San Diego

32. Glaser, S., Wu, X., Braslawsky, G., Chinn, P., Kazules, T., Morena, R., Perret, D., Hopp, J., Yang, T., Reff, M. Tumor Targeting of Tetravalent C_H2 Domain-Deleted CC49 mAbs. Journal of Immunotherapy. Volume 25, Number 6: S33, 2003.

33. Hughes, I., Chinn, P., Kazules, T., Morena, R., Perret, D., Hopp, J., Hathaway, K., Barnett, R., Reff, M., Glaser, S. Production, Characterization, and Biodistribution Studies of a Novel Hinge Engineered Domain-deleted CC49 Monoclonal Antibody. International Society for Biological Therapy of Cancer (iSBTc) Nov. 5 - 7, 2004, San Francisco, CA.

34. Hughes, I., Chinn, P., Kazules, T., Morena, R., Perret, D., Hopp, J., Hathaway, K., Barnett, R., Reff, M., Glaser, S. Production, Characterization, and Biodistribution Studies of a Novel Hinge Engineered Domain-deleted CC49 Monoclonal Antibody. 2nd annual Antibody Therapeutics Conference Nov. 30 - Dec. 3, 2004, San Diego, CA.

35. Wu, X., Hopp, J., Perret, D., Chung, J., Reff, M., Glaser, S. Generation and Characterization of Two Tetravalent CH2 Domain- deleted CC49 mAbs. International Society for Biological Therapy of Cancer (iSBTc) Nov. 5 - 7, 2004, San Francisco, CA.

36. Wu, X., Hopp, J., Perret, D., Chung, J., Reff, M., Glaser, S. Generation and Characterization of Two Tetravalent CH2 Domain- deleted CC49 mAbs. 2nd annual Antibody Therapeutics Conference Nov. 30 - Dec. 3, 2004, San Diego, CA.

37. Glaser, S., Brenneise, I., Braslawsky, G., Chinn, P., Kazules, T., Morena, R., Perret, D., Hopp, J., Wang, T., Reff, M. Novel Antibody Hinge Regions for Efficient Production of CH2 Domain-Deleted Antibodies. The Eleventh International Conference on Human Antibodies & Hybridomas October 6 -8, 2004, The Alexander Hotel, Dublin, Ireland. Human Antibodies 13, 34-35, 2005

38. Glaser, S., Brenneise, I., Braslawsky, G., Chinn, P., Kazules, T., Morena, R., Perret, D., Hopp, J., Wang, T., Reff, M. Novel Antibody Hinge Regions for Efficient Production of CH2 Domain-Deleted Antibodies. IBC's 14th Antibody Production & Downstream Processing Conference March 9-11, 2005, San Diego, CA..

EXHIBIT B

CHAPTER
24

Mammalian Expression Vectors

Anthony A.G. Ridgway

The term *vector* in molecular biology has a broad definition. It encompasses not just the vehicle by which the material for experimental investigation is introduced into a host but, since this material is often naked DNA, includes the collection of nucleotide sequences that determine the level to which the foreign information is expressed. The use of vectors to study the expression of cloned genes is a natural extension of their use in the cloning and sequencing of genes. Many genes, whose functions are known because mutants exist or because their isolation had been dependent on the expression of a certain phenotype, have been further characterized using expression vectors. However, the application of new techniques in molecular biology has generated eukaryotic cDNA clones correlated with a particular set of cellular events and about which little else, other than the DNA sequence, is known. A major challenge to the molecular biologist is to determine the biological function of the products of such "unknown clones" and their relationship with other proteins involved in the same biological process. Manipulating the expression of genes is one way of learning more about them.

There are many excellent reviews of mammalian expression vectors (Elder et al. 1981; Gluzman 1982; Gruss and Khoury 1982; Kucherlapati and Skoultchi 1984; Rigby 1982, 1983; Subramani and Southern 1983). In

this chapter I aim to complement more detailed treatises by providing an overview of the kinds of vectors available, especially those recently described. I will not discuss retroviral vectors, which are covered in Chapter 25.

24.1 SOME REQUIREMENTS OF MAMMALIAN EXPRESSION VECTORS

The primary requirements of mammalian expression vectors are the correct and efficient expression of the cloned gene. These depend on a number of sequence elements that influence the level of transcription, where it starts and stops, and whether the transcribed mRNA will be correctly processed and transported. Several discrete regions upstream from the transcriptional start site constitute the promoter. These function only if their spacing and orientation are maintained relative to the start site (Breathnach and Chambon 1981; Dierks et al. 1983; McKnight 1982). Another functional element is the transcriptional enhancer. Enhancers, by definition, can function in either orientation relative to the gene and at considerable distances (Khoury and Gruss 1983). Transcriptional termination is poorly understood; however, cleavage and subsequent addition of a poly(A) tract at the end of most messages is required for correct processing and occurs from 10 to 20 nucleotides downstream from the consensus sequence AATAAA (Birnstiel et al. 1985). There are conflicting reports regarding the value of including splicing signals in the vector (Rigby 1983). The removal of sequences that for unknown reasons interfere with processing, rather than the occurrence of a splice event itself, may have led to this confusion.

Mammalian expression vectors must be capable of replication but not necessarily in mammalian cells. Many mammalian vectors contain a suitable origin of replication and a selectable marker allowing them to be propagated as plasmids in bacteria. If the vector is very large, such as those based on adenovirus and vaccinia virus, then a fragment that can be manipulated and into which foreign DNA is inserted is propagated in a prokaryotic vector and eventually joined to the rest of the eukaryotic vector by ligation or recombination (see sections 24.8 and 24.10). Vectors that can replicate in both prokaryotic and eukaryotic cells are called *shuttle vectors*.

The recombinant vector should confer a selective advantage on the mammalian cell it inhabits. This may take the form of a replicative function that complements a defect in an otherwise replication-competent helper virus, thus ensuring the persistence of the vector. If the vector itself is replication-competent, the ability to isolate pure stocks, from a lytic plaque for example, may be sufficient, although restriction analysis and hybridization to probes may be required for initial identification. The most convenient

way is for the vector to carry a selectable marker gene. These genes fall into two categories: recessive and dominant. Recessive genes can only be used in mutant cells unable to express that gene. Examples include genes coding for thymidine kinase (TK), dihydrofolate reductase (DHFR), adenine phosphoribosyl transferase (APRT), and hypoxanthine-guanine phosphoribosyl transferase (HPRT). Use of these markers greatly limits the potential host range since isolation of cells with the appropriate phenotype can be a major undertaking. Dominant genes are those that confer resistance to an abnormal metabolic challenge and so are selectable in any cell capable of expressing them. Examples include a mutant form of DHFR that provides resistance to methotrexate (Simonsen and Levinson 1983) and two bacterial genes that, with the appropriate regulatory signals, are expressed in mammalian cells. These are *gpt* (xanthine-guanine phosphoribosyl transferase), which permits growth in mycophenolic acid if xanthine is provided as a substrate for the purine salvage pathway (Mulligan and Berg 1981), and *neo* (aminoglycoside 3′-phosphotransferase type II; an enzyme that inactivates antibiotics such as gentamycin, neomycin, and kanamycin), which provides resistance to the block in protein synthesis caused by the drug G418 (Southern and Berg 1982). Where stable transformants are to be selected, the marker need not be molecularly joined to the vector but can be cotransfected along with the vector. A proportion of the cells that have stably incorporated the marker will also have incorporated vector sequences.

The ability to accept large inserts is desirable, especially for the expression of genomic fragments. For those vectors that are not packaged into virions but are replicated as bacterial plasmids, size is not severely limiting; however, efficient replication and ease of isolation decreases with increasing size. For virion vectors, packaging limits can be stringent, and if the vector is to remain replication-competent, the extent to which vector sequences can be substituted by foreign DNA becomes a factor. If certain essential viral functions are provided in *trans* by a helper virus or by the host cell, the capacity for substitution increases accordingly.

A wide host range is a desirable characteristic. For virion vectors, host range is determined by the presence of appropriate receptors on the cell surface and, for those that depend on the host transcriptional apparatus, by the degree to which cellular transcription factors recognize the transcriptional control signals in the vector. For nonvirion vectors only the latter criterion applies. Enhancers appear to be the chief determinant of cell-type specificity (Chatis et al. 1984; DesGroseillers and Jolicoeur 1984; Hanahan 1985; Lenz et al. 1984; Lenz and Haseltine 1983; Ornitz et al. 1985), although recent evidence suggests that promoter elements can also play a role (Foster et al. 1985; Gopal et al. 1985; Mason et al. 1985). The involvement of cell-type-specific factors in transcription has been confirmed by experiments illustrating competition for such factors (Ciliberto et al. 1985; Mercola et al. 1985; Sassone-Corsi et al. 1985; Schöler and Gruss 1984).

24.2 INTRODUCTION OF VECTOR INTO HOST CELLS

There are numerous methods for transferring genetic material into mammalian cells; they may be grouped into four categories.

1. Transfection, or the administration of DNA to cells in a form that enhances its passage through the cell membrane. This category includes electrophoresis (Ocho et al. 1981), electroporation (Neumann et al. 1982), the administration of DNA-calcium phosphate precipitates to cells (Graham et al. 1980), and the treatment of cells with polycations such as DEAE dextran (Gopal 1985; McCutchan and Pagano 1968; Sussman and Milman 1984) or hexadimethrine bromide (Polybrene) (Kawai and Nishizawa 1984) before, or along with, DNA in solution. Subsequent brief treatment with agents such as glycerol (Parker and Stark 1979), dimethyl sulfoxide (DMSO) (Lewis et al. 1980; Stow and Wilkie 1976) or polyethylene glycol (PEG) (Gopal 1985) "shock" cells into taking up DNA attached to their surface and improve efficiency of transfection. Although the calcium phosphate technique was originally designed for use with naked DNA, it also works with chromatin (Klobutcher and Ruddle 1981) and bacteriophage (Ishiura et al. 1982).
2. Cell fusion with enveloped DNA. DNA packaged in bacterial protoplasts (Schaffner 1980; Rassoulzadegan et al. 1982; Sandri-Goldin et al. 1981), red cell ghosts (Boogaard and Dixon 1983), or liposomes (Hoffman et al. 1981; Schaefer-Ridder et al. 1982) is efficiently transduced by fusion with cells using methods similar to those developed to create somatic cell hybrids.
3. Microinjection. Highly efficient transfection has been achieved using microinjection directly into cell nuclei (Capecchi 1980; Diacumakos 1973; Graessmann et al. 1980). This approach has been used to introduce genes into preimplantation mouse embryos and allow the subsequent study of foreign gene expression in every cell and tissue type (Hanahan 1985; Ornitz et al. 1985).
4. Infection with intact viruses. High-level transient expression is achieved during lytic infection of permissive hosts by DNA viruses. Establishment of stable transformants by these vectors requires integration of viral DNA into the genome of a nonpermissive host or replication-defective viral DNA into a permissive host and occurs at a much lower frequency. Alternatively, stable transformants can be created through nonlytic infection by vectors that replicate episomally.

Calcium phosphate- and DEAE dextran-mediated transfer are the most frequently used procedures; the low efficiencies of transfection achieved do not usually cause a problem since in most cases selective pressure is applied. However, the cell-type and experimental situation dictate the approach. For

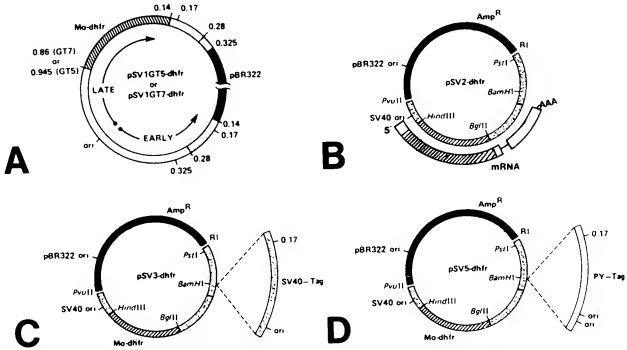


FIGURE 24–1 Some SV40-derived plasmid vectors. **A**, Late region expression vectors, pSV1GT5 (insert at map position 0.945) and pSV1GT7 (insert at map position 0.86). **B**, pSV2 containing the mouse DHFR gene. Insertion of foreign DNA occurs between the *Hin*dIII and *Bgl*II sites. Vector contribution to the mRNA is also shown (open boxes). **C** and **D**, Derivatives of pSV2 capable of replication in cells permissive for SV40 (pSV3) and polyoma (pSV5) by virtue of the inclusion of the SV40 large T and the polyoma large T antigen genes, respectively. pSV5 also contains a duplication of the polyoma virus origin of replication. Solid boxes, pBR322-derived sequences; stippled boxes, SV40-derived sequences; cross-hatched boxes, mouse DHFR gene; numbers correspond to SV40 map units. (Adapted from Subramani and Southern [1983] and Subramani et al. [1981].)

a review of methods of introducing genes into cells and their fate, see Kucherlapati and Skoultchi (1984).

24.3 PAPOVA VIRUS VECTORS

The first generation of eukaryotic cloning vehicles was based on SV40 for the simple reason that this small DNA virus was the first for which the requisite information on sequence, transcription, and replication became available (Tooze 1981). The small size of the virus and stringent packaging limits meant that not only did foreign genes have to be small, but much of the virus had to be removed to make room. Foreign genes were usually positioned so as to be under the control of the strong late region promoter, which provided higher levels of expression than the early region promoter. As long as the substitution affected only the early or the late region, a virus temperature-sensitive in the other region could be used as helper, and progeny recombinant and helper virus could be generated through complementation. This approach was used to express numerous foreign genes (for review, see Rigby 1983 and Subramani and Southern 1983). The general nomenclature SVGTX-Y is used to describe these vectors; X is a number that represents position in the genome, odd or even for late or early region, respectively; Y represents an abbreviated description of the inserted DNA (Subramani and Southern 1983). The SVGTX series was modified through a small sequence duplication to allow insertion into pBR322 (to create pSV1GTX; Figure 24–1,*A*) and propagation in *E. coli*, but infection of a mammalian host required either excision of SVGTX, circularization and transfection, or homologous recombination in vivo (within the small duplication) (Subramani and Southern 1983).

The opportunity to obtain pure recombinant virus stocks arose when viral early region functions could be provided in *trans*. The CV-1 cell line (African green monkey kidney cells), permissive for SV40 lytic infection, was transformed by Gluzman (1981) using a manipulated SV40 genome. The resulting COS (CV1-origin-SV40) cell line expresses large T antigen and factors necessary for viral replication; the resident SV40 genome is replicated along with the cellular DNA and cannot be rescued. SV40 recombinants wherein the foreign gene has been substituted for early region functions can thus be replicated and packaged in COS cells. Unfortunately, a small substitution capacity and stringent packaging constraints limit the usefulness of SV40 as a virion vector.

With the advent of improved DNA transfection techniques and positive selection markers, portions of the SV40 genome have been put to more productive use in the control of expression of genes stably introduced into cells. Stable transformants permit long-term study of gene expression rather than the massive, transient expression observed during lytic infection. If the origin of replication (*ori*) is retained, the recombinant molecule can be

replicated extrachromosomally in the presence of large-T, for example in COS cells; however, large-T antigen causes some repression of the early promoter. (See section 24.8.)

Polyoma virus has also been used as a virion vector, albeit infrequently, and has the advantage of a host range that includes murine cells. However, its use is subject to the same limitations as SV40 (Gluzman 1982).

24.4 NONRESTRICTIVE VECTORS

Vectors that are not packaged into virions and are thus free of most limitations on size and host range can be referred to as nonrestrictive; such vectors can be constructed using DNA from various prokaryotic and eukaryotic sources.

Fragments of the SV40 genome have been extensively used in plasmid vectors that can be conveniently propagated in prokaryotes (the pSV series). Purified plasmid DNA is then introduced into host cells by any of the methods discussed above. The function of the SV40 sequences is to provide the appropriate signals, recognizable by mammalian cells, for efficient transcription. Since these vectors are designed to become stably acquired by the host cell by integration into the genome, viral structural genes are not required. The prototype vector of this genre, pSV2 (Figure 24–1, *B*) contains the origin of replication and the β-lactamase gene from pBR322. The contribution from SV40 includes a eukaryotic transcription unit comprising enhancer, promoter (from the early region), an intron with splicing signals (from the small t antigen gene), and signals for polyadenylation (also from the early region). Foreign DNA is inserted between the promoter and the intron splice donor and must provide an AUG codon for initiation of translation. Polyadenylation can occur within the cloned insert or at a site downstream in the vector.

Initially, many of the genes inserted were markers whose correct expression provided the recipient cell with a selective advantage, for example, by correcting a biochemical defect in the cell (for example, TK, DHFR) or providing resistance to a drug (for example, *gpt, neo*). These selectable markers can serve as a component of the vector and provide a selective advantage for a nonselectable gene of experimental interest positioned elsewhere in the vector. This second gene would also require appropriate signals for expression, not necessarily from SV40.

pSV2 and other vectors containing the SV40 *ori* cannot replicate in their natural mammalian host, monkey cells, in the absence of large T antigen. They can, however, replicate in the large T-expressing COS cell line (Gluzman 1981) in episomal fashion (see section 24.8). pSV3 and pSV5 are derivatives of pSV2 that can replicate in monkey and mouse cells, respectively (Figure 24–1, *C* and *D*).

Episomal replication of vector sequences is one way of increasing the

copy number and degree of expression of a gene of interest. Another method is cotransfection with the amplifiable DHFR gene. Cells selected for resistance to the antifolate drug methotrexate (a competitive inhibitor of DHFR) are usually found to contain multiple copies of the wild-type DHFR gene and, consequently, large amounts of enzyme (for review, see Stark and Wahl 1984). Extensive amounts of flanking DNA also become amplified; therefore, genes colinear with an expressible DHFR gene or joined in vivo following cotransfection, also become overexpressed. This strategy has been employed to achieve high-level expression of several genes (Choo et al. 1986; Christman et al. 1982; Kaufman and Sharp 1982; Kim and Wold 1985; Ringold et al. 1982).

24.5 REGULATABLE VECTORS

The transcriptional control regions of several genes whose expression is inducible have been used in attempts to control heterologous gene expression following formation of chimeric genes. These include those of the mouse mammary tumor virus LTR (Jakobovits et al. 1984; Izant and Weintraub 1985; Ostrowski et al. 1984; Papkoff and Ringold 1984); metallothionein genes (Hamer and Walling 1982; Karin et al. 1984); heat shock genes (McMahon et al. 1984; Pelham and Bienz 1982); and interferon genes (Goodbourn et al. 1985; Ryals et al. 1985). With a few exceptions, the initial objectives have not been realized due, primarily, to higher than expected constitutive levels of transcription and variable but low levels of induction. This is likely due in part to the site of integration in the cell genome and to the copy number of the chimeric gene and availability of positive and negative transcription factors in the host cell. Consequently, vectors employing these techniques are not yet in wide use.

Another approach to regulate gene expression involves amplification through the temperature-sensitive (ts) regulation of vector replication. Two slightly different methods have recently been used. Rio et al. (1985) constructed a ts version of COS cells by placing SV40 ts mutant large T antigen gene under the direction of the highly efficient Rous sarcoma virus LTR, which is insensitive to feedback repression by T antigen. These cells support replication from the SV40 *ori* at 33°C but not at 40°C and allow regulation of the copy number of transfected SV40 *ori*-containing vectors. Selectable vector sequences can be maintained as integrated DNA or as autonomously replicating episomes by modulating T antigen activity in the ts COS cells. Portela et al. (1985) have constructed a vector for the direct cloning of cDNA of known genes that includes a different ts mutant T antigen gene. The foreign DNA is under direction of the SV40 late promoter, so at the permissive temperature both amplification and T antigen–stimulated transcription occur.

24.6 VECTORS FOR THE ANALYSIS OF SEQUENCE ELEMENTS THAT CONTROL EXPRESSION

Vectors containing dominant or recessive selectable markers can be used to analyze sequence elements that control expression, but the selection procedures are laborious and time consuming; also factors other than the efficiency of the control signals can come into play. An elegant system has been described by Gorman et al. (1982a) that allows for easy and rapid analysis of control elements using a transient expression assay. The system depends on the gene from transposon Tn9 encoding chloramphenicol acetyltransferase (CAT). The CAT vectors are based on the pSV plasmids. The SV40 promoter and enhancer have been removed from the prototype pSV2CAT to create pSV0CAT (Gorman et al. 1982a). This construct contains a unique *Hin*dIII site immediately upstream of the *cat* gene to allow insertion of other known or putative control sequences. Another modification involves removal of just the enhancer to create pSV1CAT (Gorman et al. 1982a) and $pA_{10}CAT_2$ (Laimins et al. 1984), allowing one to test the influence of putative enhancer segments on transcription from an SV40 promoter.

Use of this vector system has facilitated the dissection of transcription control signals that influence initiation and termination of transcription and has permitted comparison of the efficiency with which such signals are used in different cell types (Celander and Haseltine 1984; Derse et al. 1985; D'Onofrio et al. 1985; Gorman et al. 1982a, 1982b; Herbomel et al. 1984; Laimins et al. 1984; Overbeek et al. 1986; Sato et al. 1986; Sodroski et al. 1984). As a natural extension of such studies, enhancer-CAT chimeras are being used to analyze the binding domains and specificity of viral and cellular *trans*-acting transcription and translation factors (De Franco and Yamamoto 1986; Greene et al. 1986; Rosen et al. 1986b, 1986c; Schöler et al. 1986).

24.7 VECTORS THAT PERMIT CLONING AND EXPRESSION OF cDNA INSERTS

The cloning of cDNAs for which hybridization probes do not exist presents many difficulties. Recently, in a modification of earlier work designed to produce a library of full-length cDNA clones in a bacterial expression vector (Okayama and Berg 1982), the same authors described a plasmid vector, pcD (Figure 24–2*A*) that promotes expression of the cDNA segment in mammalian cells (Okayama and Berg 1983). Transcription occurs from the SV40 early promoter. Other SV40 signals can direct both polyadenylation, should it not occur as a result of signals in the cDNA sequence, and splicing at either of two alternative splice sites 5′ to the cDNA. One splice places the initiator AUG codon provided by the cDNA first in line from the 5′

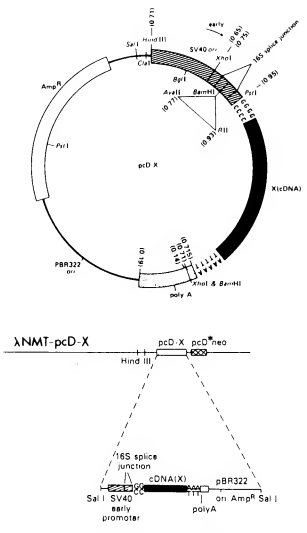


FIGURE 24–2 DNA cloning and expression vectors. **A**, pcD-X depicts the generalized structure of pcD-cDNA recombinants. The cDNA insert is bounded by a dGdC sequence at the 5′ end and a dAdT sequence at the 3′ end, both generated during the cloning procedure. Numbers refer to SV40 map positions. An alternate splice acceptor lies within the intron bounded by the 16S splice signals and is followed by an ATG translational start codon, which could give rise to a SV40-cDNA fusion protein. **B**, pcD-X can be transferred in either orientation to λ-NMT, after linearization at *Sal*I or *Hin*dIII sites, to form λ-NMT-pcD-X. Cross-hatched box, SV40 early region; solid box, cDNA insert; stippled box, SV40 late region polyadenylation signal; criss-cross-hatched box, pcD-*neo* resident in λ-NMT. (Adapted from Okayama and Berg 1983, 1985.)

end of the mRNA. The other splice event retains a vector-derived AUG upstream of the cDNA and may permit translation of a fusion protein. This vector reportedly allows the isolation of cDNAs representing mRNAs present at greater than 0.05% of the mRNA population. Difficulty in detection of rare cDNAs (those present at less than 0.05%) relates to low host-cell transformation efficiencies and elimination of potential transformants before full expression is achieved.

To increase the cloning efficiency, Okayama and Berg (1985) have developed a bacteriophage vector, λNMT, which contains *neo* as a selectable marker and into which cDNA libraries originally constructed in pcD can be transferred (Figure 24–2, *B*). Phage-mediated transfection efficiencies can reach 1% (Ishiura et al. 1982), and selection for resistance to G418 allows isolation of stable transformants, which can later be screened for specific, cDNA-encoded, functions. The application of these techniques allows the isolation of clones present in the cDNA library at a level of one functional copy per 100,000, or the equivalent of two to three copies of the mRNA per cell (Okayama and Berg 1985). The various characteristics of λNMT-pcD recombinants allow recovery of the cDNA insert by cell fusion between transduced cells and COS cells (Gluzman 1981), which promotes initiation of replication from SV40 origins and the generation of extrachromosomal circular DNA that can then be cloned directly in *E. coli* (Breitman et al. 1982).

24.8 VECTORS THAT REPLICATE EPISOMALLY

Vectors that replicate episomally are not subject to packaging restraints, and although they do not reach the high copy number of viruses that undergo lytic infection, they can be present at several hundred copies per cell. Such vectors therefore offer a potential means of studying expression from multiple copies of a large cloned insert in stable cell lines. Integration into the host cell genome is an infrequent occurrence and, except for a small proportion of concatemers and concatenates, viral genomes are usually present at 50–300 copies of single, covalently closed circles. Cloned sequences therefore do not become interrupted, as might occur on integration, and are flanked by sequences identical to those flanking all other copies of the cloned material. Any effect of vector sequences on expression will therefore be constant.

Bovine papilloma virus type 1 (BPV) has been the preferred choice for use as this kind of vector. It has a broad host range for infection and is capable of transforming rodent cells in culture. In transformed cells, expression of viral late genes and lytic viral replication do not occur. A fragment comprising 69% of the genome is sufficient for replication and transformation (Lowy et al. 1980) and was first used as a cloning vector by Sarver et al.

(1981) to introduce the rat preproinsulin gene into the C127 line of mouse fibroblasts.

The ability of the virus to induce foci of transformed mouse cells provides a marker for the isolation of cells containing the cloned material. The efficiency with which different mouse cell lines become morphologically transformed varies considerably, C127 being especially susceptible. For some cell lines, inclusion of a selectable marker in the BPV construct or cotransfection with a selectable marker is necessary. These different methods for selection of BPV-containing cell clones are comparable (Sambrook et al. 1985). The ability to propagate the vector efficiently in both mouse and bacterial cells was achieved (DiMaio et al. 1982) by linking the transforming fragment of BPV to a pBR322-derived plasmid lacking the "poison" sequences inhibitory to viral replication in eukaryotic cells (Lusky and Botchan 1981).

Different results have been reported regarding the relative expression of hepatitis B virus (HBV) antigen genes cloned in the two possible transcriptional orientations (Wang et al. 1983; Denniston et al. 1984). The high incidence of genetic rearrangement in the antigen-coding regions of some BPV-HBV recombinants in stable cell transformants possibly represented enrichment for cells no longer expressing a toxic HBV antigen rather than an intrinsic tendency of BPV vectors to undergo rearrangements (Denniston et al. 1984).

For certain purposes, for example, studying genes involved in cell transformation or control of cell cycle progression, the transforming properties of BPV may be a hindrance. Also, whereas the large copy number is advantageous in many instances and was a primary reason for adapting BPV as a vector, for certain uses it may be undesirable. An example of such a situation might be when using BPV to introduce a gene under the transcriptional control of an inducible promoter. Too many copies of such promoters usually results in excessive leakiness of transcription; for the metallothionein promoter, transcription in the absence of inducing agents (heavy metals) is thought to be reduced by a repressor present in limited quantity so that its effect is neutralized in the presence of many copies of the promoter. Therefore, cell lines with many cloned copies show high constitutive expression and poor inducibility of expression (Hsiung et al. 1984; Sambrook et al. 1985). Recently, a BPV low-copy-number mutant has been described. Only one to five copies of plasmid DNA per cell are found in cells carrying this mutant. Furthermore, this mutant is also deficient in transformation (Berg et al. 1986). The incorporation of these mutant characteristics into a BPV vector carrying a selectable marker may be useful under certain circumstances.

Fragments of SV40 and polyoma containing their origins of replication can be used as the basis of episomal vector systems. The SV40 *ori* requires large T antigen and certain other factors that can be provided in *trans* by COS cells (Gluzman 1981). Thus pBR322-derived plasmid vectors lacking

the poison sequence and containing an 85-bp fragment encompassing the SV40 *ori* (Myers and Tjian 1980) replicate very efficiently when transfected into COS cells and have been used to study the transcription of human globin genes (Mellon et al. 1981; Humphries et al. 1982). Recently, an SV40-based shuttle vector has been described that contains a minimal noninterfering bacterial component and a duplication of the SV40 enhancer and *ori* and that exhibits highly efficient expression from a SV40 late region promoter (Villarreal and Soo 1985).

These SV40-derived vectors do not replicate in mouse cells, but this can be overcome by including the polyoma early region in the vector (Mulligan and Berg 1980, 1981; Queen and Baltimore 1983; Stafford and Queen 1983). SV40 and polyoma episomal vectors replicate to a very high copy number (50,000 to 400,000 copies per cell) but are only suitable for transient analysis; perhaps because such large amounts of extrachromosomal DNA are toxic, stable cell lines do not result (Rigby 1983).

24.9 ADENOVIRUS VECTORS

An understanding of the molecular biology of the adenoviruses has lagged behind that of SV40 and the retroviruses, as has their development as vectors. The adenovirus genome is linear and approximately 36 kb. The size and the consequent presence of multiple restriction endonuclease sites presented initial problems in the manipulation of the genome. These were alleviated by the isolation, after several cycles of restriction and religation, of variants lacking designated restriction sites (Jones and Shenk 1978, 1979).

As the adenoviruses become better characterized they become more attractive as vectors and for certain specialized uses possess advantages. They exhibit a broad host range, grow to high titers during lytic infection, and appear to require only a relatively small proportion of their genome in *cis* (Tooze 1981). This latter point means that once "helper" cell lines are developed that can provide necessary viral functions in *trans*, much of the genome can be substituted. This allows room for various "on-board" features such as selectable markers and replication functions that allow propagation of part of the vector in other hosts. One use might be in the study of differential splicing events, for example.

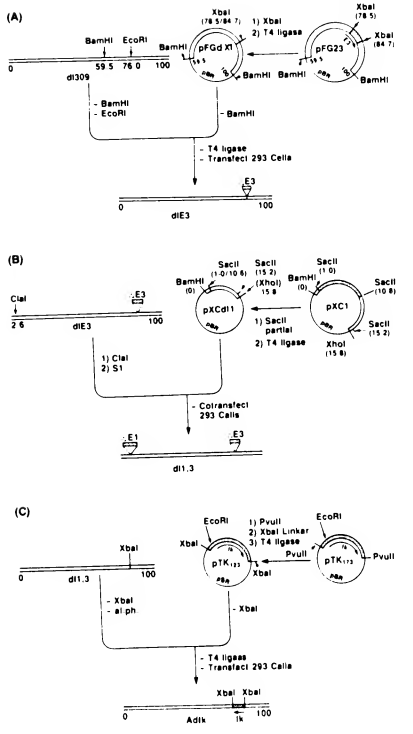
At present, only the E1 functions are available in *trans*, from strain 293 cells—human embryonic kidney cells transformed by sheared adenovirus 5 DNA (Graham et al. 1977). These functions are contained in the left-terminal 12% of the genome and are therefore conveniently placed for manipulation (see following paragraph), but allow for substitutions of only a few kilobases. The E3 region is nonessential for adenovirus replication in cultured cells, is close to the right terminus of the genome, and can be replaced to allow the study of heterologous sequences (Anderson et al. 1976; Berkner and Sharp 1983; Kelly and Lewis 1973; Saito et al. 1985). The

deletion of E3 (about 2 kb), E1, and the ability of the virus to package 38 kb sets the current upper limit for DNA inserts at approximately 7.5 kb for a helper-independent adenovirus vector able to grow lytically in strain 293 cells (Haj-Ahmad and Graham 1986).

Alteration of the adenovirus genome for use as a vector usually entails manipulation of sequences from one end of the virus in a prokaryotic vector followed by ligation to the rest of the genome after restriction of purified adenovirus DNA at a unique site. The construct is then transfected into a suitable host (Figure 24–3,*A*). Alternatively, fragments containing manipulated sequences, together with the rest of the genome, can be cotransfected so that recombination in vivo produces the required, replication-competent, construct (Figure 24–3,*B*) (Stow 1981, 1982; Gluzman 1982; Haj-Ahmed and Graham 1986). The adenovirus genome is bounded by inverted repeat elements, either of which can be regenerated during replication provided the other is intact. Therefore, prior removal of plasmid vector sequences from recombinant adenovirus fragments is not required (Stow 1982).

Helper-dependent vectors can also be used and allow for much larger substitutions of the genome. To retain the recombinant, some form of selective pressure is required. This can be achieved by including coding sequences for SV40 large T antigen in the vector and propagating the virus in monkey cells. The infection of monkey cells by human adenoviruses is abortive in the absence of large T expression. This approach was used initially to overproduce SV40 early protcins, but the strategy is general and has been used to obtain viruses carrying other unselected genes (Thummel et al. 1981, 1982, 1983; Solnick 1981; Mansour et al. 1985; Yamada et al. 1985). The vector is only ever present as a small percentage (often less than 5%) of the wild-type virus, which provides the replicative and packaging functions, but since host protein synthesis is shut down in the late stages of

FIGURE 24–3 Construction of adenovirus vectors. **A**, Strategy for construction of dlE3. pFG23 (containing the right end of adenovirus) was digested with *Xba*I and then religated to generate pFGdX1 with a deletion of most of E3. pFGdX1 was then digested with *Bam*HI and ligated to *Bam*HI$^-$ and *Eco*RI-digested strain dl309 viral DNA. d1309 contains a single *Xba*I site and a small deletion at map coordinates 3.7 and 0.86, respectively. The ligation mixture was used to transfect strain 293 cells. The *Eco*RI digestion hinders reformation of dl309. **B**, Strategy for construction of dlE1,3. pXC1 containing the left 15.8% of the adenovirus 5 genome was cut with *Sac*II (partial digestion) followed by religation to generate pXCdl1, lacking essentially all of E1. Cotransfection of strain 293 cells with a mixture of *Cla*I-digested dlE3 DNA and undigested pXCdl1 DNA yielded dlE1,3 by in vivo recombination. **C**, Construction of Adtk. pTK173 contains the entire herpes simplex virus TK gene (*Pvu*II fragment). The flanking *Pvu*II sites were converted to *Xba*I sites using linkers to create pTK123. The TK gene was then inserted into the unique *Xba*I site of dlE1,3 via a trimolecular ligation reaction following digestion of pTK123 and dlE1,3 with *Xba*I. (Adapted from Haj-Ahmad and Graham 1986.)


(A)
BamHI
EcoRI
0
59.5
76.0
100
dl309
- BamHI
- EcoRI
XbaI (78.5/84.7)
1) XbaI
2) T4 ligase
pFGd X1
BamHI
BamHI
pFG23
XbaI (78.5)
XbaI (84.7)
BamHI
BamHI
- BamHI
- T4 ligase
- Transfect 293 Cells
E3
0
100
dlE3
(B)
ClaI
2.6
E3
dlE3
100
1) ClaI
2) S1
SacII (1.0/10.5)
SacII (15.2)
BamHI (0)
(XhoI) 15.8
pXCd1
1) SacII partial
2) T4 ligase
SacII (1.0)
BamHI (0)
pXC1
SacII (10.8)
SacII (15.2)
XhoI (15.8)
- Cotransfect 293 Cells
E1
E3
dl1.3
(C)
XbaI
0
dl1.3
100
- XbaI
- alph
EcoRI
XbaI
pTK123
XbaI
1) PvuII
2) XbaI Linker
3) T4 ligase
PvuII
EcoRI
pTK123
PvuII
- XbaI
- T4 ligase
- Transfect 293 Cells
XbaI XbaI
0
Adtk
tk
100

lytic infection, RNA and protein products from the recombinant are easily identified. The greatest levels of heterologous protein are obtained from sequences expressed from the major late promoter and containing the complete tripartite leader, which enhances the efficiency of translation late in infection (Thummel et al. 1983; Logan and Shenk 1984).

Lytic infection using either helper-independent or helper-dependent adenovirus vectors has been used with success to study gene expression of viruses for which in vitro propagation techniques have not yet been optimized or whose proteins are normally present at very low levels. Besides the SV40 early genes, examples include the following: polyoma small, middle, and large T antigens (Mansour et al. 1985); herpes simplex virus thymidine kinase gene (Yamada et al. 1985); and hepatitis B virus antigens (Saito et al., 1985, 1986). The α-subunit of human chorionic gonadotropin has also been produced in such a vector system with high-level synthesis of a protein that was glycosylated and secreted (Yamada et al. 1985).

In cells nonpermissive for replication, or in permissive cells infected with replication-defective viruses, stable transformation is characterized by integration of one to several complete or almost complete copies of the genome, independently of the multiplicity of infection (Doerfler 1982; Fisher et al. 1982; Karlsson et al. 1985; Van Doren et al. 1984). In the absence of E1 products the viral genome is essentially silent, so expression of heterologous sequences is not affected by viral gene transcription. By using the E1 and E3 locations (see Figure 24–3) two different transcription units can be placed in the vector so they are separated by about 30 kb, thus reducing the probability of epigenetic suppression. (Epigenetic suppression describes the circumstance that one of two closely positioned transcription units is often expressed poorly relative to the other [Emerman and Temin 1984, 1986].)

The efficiency with which adenoviral genomes become integrated in the host genome and their physical arrangement seem to be determined primarily by the nature of the host cell and is partly related to the competence of the virus to express its own genome (Haj-Ahmad and Graham 1986; Van Doren et al. 1984). At high multiplicities of infection the efficiency of stable phenotypic transformation approaches 1% (Haj-Ahmad and Graham 1986; Van Doren et al. 1984) but can be much lower (Karlsson et al. 1985).

24.10 PARVOVIRUS VECTORS

Parvoviruses possess a linear, single-stranded DNA genome of approximately 5 kb. The most studied member of this group is adenoassociated virus (AAV), a defective human virus that requires helper functions from adenovirus or herpes simplex virus for lytic growth (Berns 1983). In the absence of helper virus, AAV can persist as an integrated provirus (Berns et al. 1975; Cheung et al. 1980; Handa et al. 1977), which is formed by recombination between AAV termini and host sequences in a manner that

leaves most AAV sequences intact. The provirus can be rescued into a productive lytic cycle by superinfection with helper (Cheung et al. 1980; Handa et al. 1977). This rescue phenomenon also occurs from unintegrated, transfected DNA; hence AAV sequences cloned into prokaryotic plasmids can be infectious (Samulski et al. 1982). The packaging limit is about 5 kb, and since the 145-bp terminal repeats are the only *cis*-acting regions needed for rescue and replication of the provirus, the substitution capacity is also approximately 5 kb. However, for the generation of single-stranded, progeny virus and for packaging the genome, viral-encoded functions are required. Therefore, substitution of AAV sequences with cloned sequences requires that both AAV adenovirus helper functions be provided in *trans*. Introduction of DNA in excess of 1 kb into a nonessential region of wild-type AAV makes the genome too large to be packaged. Thus, by cotransfecting such a construct along with the vector, one can in theory provide the required helper functions without production of wild-type particles. In practice, however, wild-type genomes are generated by recombination at a fairly high frequency (Hermonat and Muzyczka 1984). The development of a replication-competent parvovirus helper would obviate the need for adenovirus helper functions but would create additional problems related to potential pathogenic effects (Berns 1983).

Recently, an AAV-derived vector has been used to transduce the dominant selectable marker for neomycin resistance (*neo*) into human and mouse cells (Hermonat and Muzyczka 1984). The transduction frequencies were 0.4%–10% in human cells and somewhat lower in murine cells and were relatively constant with respect to multiplicity of infection. Integrated AAV genomes are normally silent in the absence of helper, thus *neo* was placed under direction of the SV40 early promoter. Transcriptional silence of vector sequences means less interference with expression of the substituted gene. AAV-derived vectors have also been used to package, transduce, and transiently express the *cat* gene (Tratschin et al. 1984). CAT expression was under the direction of endogenous promoters and required adenovirus helper functions for optimal expression.

Despite certain useful characteristics, the use of AAV as a vector has several drawbacks, the most obvious of which are the need for both AAV and adenovirus helper functions and the fact that recombinant vectors are very hard to titer since they cannot form plaques by themselves. However, the lack of any disease associated with AAV (Berns 1983) and the fact that integrated AAV genomes are stable in tissue culture for greater than 100 passages (Cheung et al. 1980) may eventually make it a viable option for certain specialized attempts at gene therapy.

24.11 VACCINIA VIRUS VECTORS

With a genome size of 187 kb (Geshlin and Berns 1974), the noninfectious nature of isolated DNA (Sam and Dumbell 1981), unique transcriptional

regulatory signals recognized by a viral RNA polymerase (Puckett and Moss 1983; Venkatesan et al. 1981, 1982; Weir and Moss 1983), and the cytoplasmic residence of infecting virus (for review see Dales and Pogo 1981), the usefulness of vaccinia virus as a vector is not immediately apparent. However, as a tool for studying the antigenicity of foreign viral proteins and, further, as an immunizing agent, it is attractive.

Vaccinia virus was successfully used as a live vaccine to eradicate smallpox and is the most thoroughly studied member of the poxvirus family. Viral recombinants were shown to be capable of expressing the cloned herpes simplex thymidine kinase (TK) gene in TK-deficient variants of vaccinia (Mackett et al. 1982; Panicali and Paoletti 1982). Recombinants expressing a great variety of heterologous virus antigens and capable of immunizing animals against infections with influenza virus (Smith et al. 1983b); herpes simplex virus types 1 (Cremer et al. 1985; Paoletti et al. 1984) and 2 (Cremer et al. 1985); hepatitis B virus (Moss et al. 1984); rabies virus (Wiktor et al. 1984); vesicular stomatitis virus (Mackett et al. 1985); and human respiratory syncytial virus (Elango et al. 1986) have since been created.

Criteria that favor vaccinia's use as a vector include a wide host range and the absence of stringent packaging restraints. At least 25 kb of exogenous DNA can be accommodated without loss of infectivity (Smith and Moss 1983), and this amount could be much larger if regions of the virus known to be nonessential for replication were deleted (Moss et al. 1981; Panicali et al. 1981).

Introduction of foreign DNA into the viral genome involves site-directed recombination in vivo. First, recombinant DNA techniques are used to construct a plasmid containing a chimeric gene flanked by vaccinia virus DNA. This is introduced by transfection into cells infected by vaccinia virus. Recombination occurs with the site of insertion determined by the flanking DNA sequences present in the plasmid. This site must be a region nonessential for replication. Two approaches are used (Mackett et al. 1982; Panicali and Paoletti 1982); disruption of the genome at the site of recombination may allow for selection based on the loss of a viral function, or the inserted DNA may carry a selectable marker. A preferred site for insertion exists within the gene for thymidine kinase; TK^- recombinants are selected by plaque assay in the presence of bromodeoxyuridine. To distinguish recombinants from spontaneous TK^- mutants, hybridization to a radiolabeled probe is required. Alternatively, a plasmid containing both a functional TK gene from herpes simplex virus and a gene of experimental interest (and both flanked with appropriate vaccinia sequences) is transfected into TK^- cells infected with a TK^- mutant of vaccinia virus, and TK^+ recombinants are isolated after growth in selective medium.

Several factors related to the cytoplasmic site of transcription must be considered when expressing genes in vaccinia vectors. Efficient expression depends on the use of vaccinia virus transcriptional regulatory signals that are distinct from prokaryotic and eukaryotic consensus sequences and are

recognized by the viral RNA polymerase (Puckett and Moss 1983; Venkatesan et al. 1981; Weir and Moss 1983). There are no indications that vaccinia RNA is spliced, and only expression of continuous protein-coding sequences has been demonstrated. To facilitate the use of vaccinia as a cloning and expression vehicle, plasmid vectors have been constructed that contain restriction sites for the insertion of foreign coding sequences, defined promoters, and flanking segments of the vaccinia virus TK gene to direct recombination. The foreign genes must retain their translational start signals (Mackett et al. 1984).

More recently, modified vectors have been described that allow rapid discrimination between TK^- recombinants and spontaneous TK^- mutants and that can actually be used as an independent indicator of recombination. These vectors incorporate the *E. coli lacZ* gene, which encodes β-galactosidase (Figure 24–4). Expression of this enzyme colors lytic plaques blue in the presence of an appropriate substrate. In prokaryotic expression vectors, retention or loss of enzyme activity provides an indication of whether insertion of DNA occurred in frame or out of frame with the *lacZ* gene (Casadaban et al. 1983; Messing et al. 1980). In vaccinia vectors, expression of the *lacZ* gene provides a marker for incorporation into the vaccinia genome

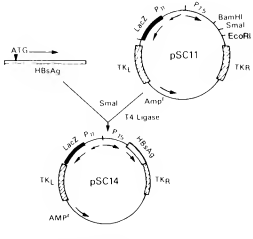


FIGURE 24–4 A vaccinia virus coexpression vector. pSC11 contains the *lacZ* gene under direction of the vaccinia P_{11} promoter and several unique sites downstream of the vaccinia $P_{7.5}$ promoter suitable for insertion of foreign DNA. In the example shown, a blunt-ended DNA fragment containing the coding sequence of the hepatitis B virus surface antigen (HBsAg) was inserted into the *SmaI* site of pSC11 to create pSC14. Plasmid DNA is transfected into vaccinia-infected cells, and the *lacZ* and HBsAg transcription units are introduced into the vaccinia virus genome by homologous recombination in vivo directed by flanking segments of vaccinia TK gene DNA. Arrows denote direction of transcription. (Adapted from Chakrabarti et al. 1985.)

of a recombinant "cassette" containing a linked gene of experimental interest (Chakrabarti et al. 1985). Since, under the conditions described, recombinants occur with a frequency of about 10^{-3}, screening can be carried out in the absence of TK^- selection, allowing greater flexibility in the choice of viral integration site and in the use of cells that are not TK^-.

In an alternate approach, a vaccinia virus recombinant expressing β-galactosidase was used as the parental virus, and new recombinants in which the β-galactosidase gene was replaccd by a gene of experimental interest were identified by the formation of colorless plaques (Chakrabarti et al. 1985).

These recent advances greatly simplify the use of this expression system. The products of expression, where carefully analyzed, have been found to be identical to the natural product, incorporating correct posttranslational modifications (Cremer et al. 1985; Mackett et al. 1985; Smith et al. 1983a, 1983b; Wiktor et al. 1984; Elango et al. 1986). This fidelity of expression and consequent correct membrane presentation of heterologous viral antigens perhaps explains how certain recombinants are capable of stimulating a cytotoxic T-lymphocyte response (Bennink et al. 1984; Yewdell et al. 1985). This demonstrates the potential of the system as a tool to identify viral antigens capable of inducing a cell-mediated immune response. However, vaccinia virus vectors show an even greater potential as a source of live vaccines, particularly in the Third World, where stability and lack of expense are prime considerations (Smith et al. 1983a; Paoletti et al. 1984). Veterinary use is a strong possibility (Mackett et al. 1985). Finally, the capacity of the recombinants to accept large amounts of foreign DNA suggests the possibility of developing polyvalent vaccines (Smith et al. 1983b; Paoletti et al. 1984).

24.12 WHAT THE FUTURE MAY HOLD

Recent developments in molecular biology that contribute to an understanding of the control of gene expression will eventually be incorporated into mammalian expression vectors. This is a necessity if we are to be able to manipulate the expression of endogenous and exogenously acquired genes in a fashion that will reveal the function and significance of their encoded proteins.

Of the many viral systems recently described that exhibit *trans*-activation of gene expression by viral genc (*tat* gene) products, the greatest levels of activation described (several hundredfold to over a thousandfold) are those for human T-cell leukemia virus (HTLV) −I and −II; human T-cell lymphotrophic virus/lymphadenopathy-associated virus (HTLV-III/LAV) or human immunodeficiency virus (HIV; Coffin et al. 1986); and bovine leukemia virus (BLV) (Rosen et al. 1985; Sodroski et al. 1984, 1985). The *tat* gene products recognize sequence elements in the retroviral LTRs. For HIV these sequences form part of the mRNA, and stimulation is posttranscriptional

(Rosen et al. 1986b); some evidence suggests this may also be the case for BLV (Derse et al. 1985). BLV *tat*-mediated expression likely also involves a transcriptional component, and for HTLV-I and −II, stimulation is clearly at the level of transcription (Rosen et al. 1986c). Incorporation of these LTRs, or appropriate regions thereof, into vectors will allow high-level expression of inserted genes in cells where the *tat* genes are also expressed. Cell lines constitutively expressing *tat* genes have recently been derived (Rosen et al. 1986a). If *tat* gene expression can be stringently controlled through inducible promoters, the prospects are vast. This methodology would be particularly useful for shutting down endogenous gene expression through high levels of antisense RNA (Kim and Wold 1985; Weintraub et al. 1985).

The use of inducible promoters in expression vectors has not reached its full potential due to constitutive levels of transcription and levels of induction that are, respectively, higher and lower than expected. Recently, the prokaryotic *LexA* repressor protein was shown to repress transcription from a yeast transcriptional control region into which the *LexA* operator had been inserted (Brent and Ptashne 1984). Other bacterial genes (*neo, gpt, cat*) can be efficiently expressed in mammalian cells, so we may see some prokaryotic regulators of gene expression employed in attempts to manipulate expression of cloned genes in mammalian systems.

The potential for fine "tailoring" of vectors for specific purposes is improving, and an ever increasing number of options is available. By bracketing a gene of interest between 5′ and 3′ untranslated regions of the c-*fos* or histone H3 genes, it is possible to direct the expression of transfected DNA to a particular time period during the cell cycle and influence the half-life of the mRNA (Artishevsky et al. 1985; Treisman 1985). The opportunity now exists to direct expression of transduced genes to a particular cell population in an animal by using transcriptional control regions that exhibit such specificity. The metal-responsive element of the metallothionein promoter and the glucocorticoid-responsive element of the Moloney murinc leukemia virus LTR (not to be confused with mouse mammary tumor virus) are distinct from the other transcription control elements of these two genes, and it should be possible to "mix and match" these with other control elements to produce novel regulatory units (DeFranco and Yamamoto 1986; Schöler et al. 1986).

Finally, recent successes at targeting genes to specific sites in the mammalian genome provides the means to generate desired mutations in a cloned cDNA and then introducc the mutation into the genome (Thomas et al. 1986). The prospects for gene therapy are obvious.

REFERENCES

Anderson, C.W., Lewis, J.B., Baum, P.R., and Gesteland, R.F. (1976) *J. Virol.* 18, 685–692.

Artishevsky, A., Grafsky, A., and Lee, A.S. (1985) *Science* 230, 1061–1063.

Bennink, J.R., Yewdell, W., Smith, G.L., Moller, C., and Moss, B. (1984) *Nature* 311, 578–579.
Berg, L.J., Singh, K., and Botchan, M. (1986) *Mol. Cell. Biol.* 6, 859–869.
Berkner, K.L., and Sharp, P.A. (1983) *Nucl. Acids Res.* 11, 6003–6020.
Berns, K.I., ed. (1983) *The Parvoviruses,* Plenum Press, New York and London.
Berns, K.I., Pinkerton, T.C., Thomas, G.F., and Hoggan, M.D. (1975) *Virology* 68, 556–560.
Birnstiel, M.L., Busslinger, M., and Strub, K. (1985) *Cell* 41, 349–359.
Boogaard, C., and Dixon, G.H. (1983) *Exp. Cell Res.* 143, 175–190.
Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* 50, 349–383.
Breitman, M.L., Tsui, L.C., Buchwald, M., and Siminovitch, L. (1982) *Mol. Cell. Biol.* 2, 966–976.
Brent, R., and Ptashne, M. (1984) *Nature* 312, 612–615.
Capecchi, M.E. (1980) *Cell* 22, 479–488.
Casadaban, M.J., Martinez-Arias, A., Shapiro, S.K., and Chou, J. (1983) *Methods Enzymol.* 100, 293–308.
Celander, D., and Haseltine, W.A. (1984) *Nature* 312, 159–162.
Chakrabarti, S., Brechling, K., and Moss, B. (1985) *Mol. Cell. Biol.* 5, 3403–3409.
Chatis, P.A., Holland, C.A., Silver, J.E., Frederickson, T.N., Hopkins, N., and Hartley, J. (1984) *J. Virol.* 52, 248–254.
Cheung, A.K.-M., Hoggan, M.D., Hauswirth, W.W., and Berns, K.I. (1980) *J. Virol.* 33, 739–748.
Choo, K.H., Filby, R.G., Jennings, I.G., Peterson, G., and Fowler, K. (1986) *DNA* 5, 529–537.
Christman, J.K., Gerber, M., Price, P.M., Flordellis, C., Edelman, J., and Acs, G. (1982) *Proc. Natl. Acad. Sci.* 79, 1815–1819.
Ciliberto, G., Dente, L., and Cortese, R. (1985) *Cell* 41, 531–540.
Coffin, J., Haase, A., Levy J.A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and Weiss, R. (1986) *Nature* 321, 10.
Cremer, K.J., Mackett, M., Wohlenberg, C., Notkins, A.L., and Moss, B. (1985) *Science* 228, 737–739.
Dales, S., and Pogo, B.G.T. (1981) *Virol. Monogr.* 18, 1–109.
DeFranco, D., and Yamamoto, K.R. (1986) *Mol. Cell. Biol.* 6, 993–1001.
Denniston, K.J., Yoneyama, T., Hoyer, B.H., and Gerin, J.L. (1984) *Gene* 32, 357–368.
Derse, D., Caradonna, S., and Casey, J.W. (1985) *Science* 227, 317–320.
DesGroseillers, L., and Jolicoeur, P. (1984) *J. Virol.* 52, 945–952.
Diacumakos, E.G. (1973) *Methods Cell Biol.* 7, 287–311.
Dierks, P., van Ooyen, A., Cochran, M.D., Dobkin, C., Reisser, J., and Weissman, C. (1983) *Cell* 32, 695–706.
DiMaio, D., Treisman, R., and Maniatis, T. (1982) *Proc. Natl. Acad. Sci.* 79, 4030–4034.
Doerfler, W. (1982) *Curr. Top. Microbiol. Immunol.* 101, 127–194.
D'Onofrio, C., Colantuoni, V., and Cortese, R. (1985) *EMBO J.* 4, 1981–1989.
Elango, N., Prince, G.A., Murphy, B.R., Venkatesan, S., Chanock, R.M., and Moss, B. (1986) *Proc. Natl. Acad. Sci.* 83, 1906–1910.
Elder, J.T., Spritz, R.A., and Weissman, S.M. (1981) *Annu. Rev. Genet.* 15, 295–340.

Emerman, M., and Temin, H.M. (1984) *Cell* 39, 459–467.
Emerman, M., and Temin, H. (1986) *Mol. Cell. Biol.* 6, 782–800.
Fisher, P.B., Babiss, L.E., Weinstein, I.B., and Ginsberg, H.S. (1982) *Proc. Natl. Acad. Sci.* 79, 3527–3531.
Foster, J., Stafford, J., and Queen, C. (1985) *Nature* 315, 423–425.
Geshlin, P., and Berns, K.I. (1974) *J. Mol. Biol.* 88, 785–796.
Gluzman, Y. (1981) *Cell* 23, 175–182.
Gluzman, Y., ed. (1982) *Eukaryotic Viral Vectors,* Cold Spring Harbor Laboratory, New York.
Goodbourn, S., Zinn, K., and Maniatis, T. (1985) *Cell* 41, 509–520.
Gopal, T.V. (1985) *Mol. Cell. Biol.* 5, 1188–1190.
Gopal, T.V., Shimada, T., Baur, A.W., and Nienhuis, A.W. (1985) *Science* 229, 1102–1104.
Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H. (1982a) *Proc. Natl. Acad. Sci.* 79, 6777–6781.
Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982b) *Mol. Cell. Biol.* 2, 1044–1051.
Graessmann, A., Graessman, M., and Mueller, C. (1980) *Methods Enzymol.* 65, 816–825.
Graham, F.L., Bacchetti, S., McKinnon, R., Stanners, C., Cordell, B., and Goodman, H. (1980) in *Introduction of Macromolecules into Viable Mammalian Cells* (Baserga, R., Croce, C., and Rovera, G., eds.) pp. 3–25, Alan R. Liss, New York.
Graham, G.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) *J. Gen. Virol.* 36, 59–72.
Greene, W.C., Leonard, W.J., Wano, Y., Svetlik, P.B., Peffer, N.J., Sodroski, J.G., Rosen, C.A., Goh, W.C., and Haseltine, W.A. (1986) *Science* 232, 877–880.
Gruss, P., and Khoury, G. (1982) *Curr. Top. Microbiol. Immunol.* 96, 159–170.
Haj-Ahmad, Y., and Graham, F.L. (1986) *J. Virol.* 57, 267–276.
Hamer, D.H., and Walling, M.-J. (1982) *J. Mol. Appl. Genet.* 1, 273–288.
Hanahan, D. (1985) *Nature* 315, 115–122.
Handa, H., Shiroki, K., and Shimojo, H. (1977) *Virology.* 82, 84–92.
Herbomel, P., Bourachot, B., and Yaniv, M. (1984) *Cell* 39, 653–662.
Hermonat, P.L., and Muzyczka, N. (1984) *Proc. Natl. Acad. Sci.* 81, 6466–6470.
Hoffman, W.K., Lalley, P., DeButler, J., Orloff, S., Schulman, S.D., and Mukherjee, A.B. (1981) *In Vitro* 17, 735–740.
Hsiung, N., Fitts, R., Wilson, S., Milne, A. and Hamer, D. (1984) *J. Mol. Appl. Genet.* 2, 497–506.
Humphries, R.K., Ley, T., Turner, P., Moulton, A.D., and Neinhuis, A.W. (1982) *Cell* 30, 173–183.
Ishiura, M., Hirose, S., Uchida, T., Hamada, Y., Suzuki, Y., and Okada, Y. (1982) *Mol. Cell. Biol.* 2, 607–616.
Izant, J.G., and Weintraub, H. (1985) *Science* 229, 345–352.
Jakobovits, E.B., Majors, J.E., and Varmus, H.E. (1984) *Cell* 38, 757–765.
Jones, N., and Shenk, T. (1978) *Cell* 13, 181–188.
Jones, N., and Shenk, T. (1979) *Cell* 17, 683–689.
Karin, M., Haslinger, A., Holtgreve, H., Cathala, G., Slater, E., and Baxter, J.D. (1984) *Cell* 36, 371–379.

Karlsson, S., Humphries, R.K., Gluzman, Y., and Nienhuis, A.W. (1985) *Proc. Natl. Acad. Sci.* 82, 158–162.
Kaufman, R.J., and Sharp, P.A. (1982) *J. Mol. Biol.* 159, 601–621.
Kawai, S., and Nishizawa, M. (1984) *Mol. Cell. Biol.* 4, 1172–1174.
Kelly, T.J., and Lewis, A.M. (1973) *J. Virol.* 12, 643–652.
Khoury, G., and Gruss, P. (1983) *Cell* 33, 313–316.
Kim, S.K., and Wold, B.J. (1985) *Cell* 42, 129–138.
Klobutcher, L.A., and Ruddle, F.H. (1981) *Annu. Rev. Biochem.* 50, 533–554.
Kucherlapati, R., and Skoultchi, A.I. (1984) *CRC Crit. Rev. Biochem.* 16, 349–379.
Laimins, L.A., Gruss, P., Pozzatti, R., Khoury, G. (1984) *J. Virol.* 49, 183–189.
Lenz, J., Celander, D., Crowther, R.L., Patarca, R., Perkins, D.W., and Haseltine, W.A. (1984) *Nature* 308, 467–470.
Lenz, J., and Haseltine, W.A. (1983) *J. Virol.* 47, 317–328.
Lewis, W.H., Srinivasan, P.R., Stokoe, N., and Siminovitch, L. (1980) *Somatic Cell Genet.* 6, 333–348.
Logan, J., and Shenk, T. (1984) *Proc. Natl. Acad. Sci.* 81, 3655–3659.
Lowy, D.R., Dvoretky, I., Shober, R., Law, M.-F., Engel, L., and Howley, P.M. (1980) *Nature* 287, 72–74.
Lusky, M., and Botchan, M. (1981) *Nature* 293, 79–81.
Mackett, M., Smith, G.L., and Moss, B. (1982) *Proc. Natl. Acad. Sci.* 79, 7415–7419.
Mackett, M., Smith, G.L., and Moss, B. (1984) *J. Virol.* 49, 857–864.
Mackett, M., Yilma, T.Y., Rose, J.A., and Boss, B. (1985) *Science* 227, 433–435.
Mansour, S.L., Grodzicker, T., and Tjian, R. (1985) *Proc. Natl. Acad. Sci.* 82, 1359–1363.
Mason, J.D., Williams, G.T., and Neuberger, M.S. (1985) *Cell* 41, 479–487.
McCutchan, J.H., and Pagano, J.S. (1968) *J. Natl. Cancer Inst.* 41, 351–356.
McKnight, S.L. (1982) *Cell* 31, 355–365.
McMahon, A.P. Novak, T.J., Britten, R.J., and Davidson, E.H. (1984) *Proc. Natl. Acad. Sci.* 81, 7490–7494.
Mellon, P., Parker, V., Gluzman, Y., and Maniatis, T. (1981) *Cell* 27, 279–288.
Mercola, M., Goverman, J., Mirell, C., and Calame, K. (1985) *Science* 227, 266–270.
Messing, J., Crea, R., and Seeburg, P.H. (1980) *Nucl. Acids Res.* 9, 309–321.
Moss, B., Smith, G.L., Gerin, J.L., and Purcell, R. (1984) *Nature* 311, 67–69.
Moss, B., Winters, E., and Cooper, J.A. (1981) *J. Virol.* 40, 387–395.
Mulligan, R.C., and Berg, P. (1980) *Science* 209, 1422–1427.
Mulligan, R.C., and Berg, P. (1981) *Proc. Natl. Acad. Sci.* 78, 2072–2076.
Myers, R.M., and Tjian, R. (1980) *Proc. Natl. Acad. Sci.* 77, 6491–6495.
Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P.H. (1982) *EMBO J.* 1, 841–845.
Ocho, M., Nakai, S., Tasaka, K., Watanabe, S., and Oda, T. (1981) *Acta Med. Okayama* 35, 381–384.
Okayama, H., and Berg, P. (1982) *Mol. Cell. Biol.* 2, 161–170.
Okayama, H., and Berg, P. (1983) *Mol. Cell. Biol.* 3, 280–289.
Okayama, H., and Berg, P. (1985) *Mol. Cell. Biol.* 5, 1136–1142.
Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., and MacDonald, R.J. (1985) *Nature* 313, 600–602.
Ostrowski, M.C., Huang, A.L., Kessel, M., Wolford, R.G., and Hager, G.L. (1984) *EMBO J.* 3, 1891–1899.

Overbeek, P.A., Lai, S.-P., Van Quill, K.R., and Westphal, H. (1986) *Science* 231, 1574–1577.
Panicali, D., Davis, S.W., Mercer, S.R., and Paoletti, E. (1981) *J. Virol.* 37, 1000–1010.
Panicali, D., and Paoletti, E. (1982) *Proc. Natl. Acad. Sci.* 79, 4927–4931.
Papkoff, J., and Ringold, G. (1984) *J. Virol.* 52, 420–430.
Paoletti, E., Lipinskas, B.R., Samsonoff, C., Mercer, S., and Panicali, D. (1984) *Proc. Natl. Acad. Sci.* 81, 193–197.
Parker, B., and Stark, G. (1979) *J. Virol.* 31, 360–369.
Pelham, H.R.B., and Bienz, M. (1982) *EMBO J.* 1, 1473–1477.
Portela, A., Melero, J.A., Martinez, C., Domingo, E., and Ortin, J. (1985) *Nucl. Acids Res.* 13, 7959–7977.
Puckett, C. and Moss, B. (1983) *Cell* 35, 441–448.
Queen, C., and Baltimore, D. (1983) *Cell* 33, 741–748.
Rassoulzadegan, M., Binetruy, B., and Cuzin, F. (1982) *Nature* 295, 257–259.
Rigby, P.W.J. (1982) in *Genetic Engineering*, Vol. 3 (Williamson, R., ed.) Academic Press, New York and London.
Rigby, P.W.J. (1983) *J. Gen. Virol.* 64, 255–266.
Ringold, G., Dieckmann, B., and Lee, F. (1982) *J. Mol. Appl. Genet.* 1, 165–175.
Rio, C.D., Clark, S.G., and Tjian, R. (1985) *Science* 227, 23–28.
Rosen, C., Sodroski, J.G., Campbell, K., and Haseltine, W.A. (1986a) *J. Virol.* 57, 379–384.
Rosen, C.A., Sodroski, J.G., Goh, W.C., Cayton, A.I., Lippke, J. and Haseltine, W.A. (1986b) *Nature* 319, 555–559
Rosen, C.A., Sodroski, J.G., Kettman, R., Burny, A., and Haseltine, W.A. (1985) *Science* 227, 320–322.
Rosen, C.A., Sodroski, J.G., Kettman, R., and Haseltine, W.A. (1986c) *J. Virol.* 57, 738–744.
Ryals, J., Dicrks, P., Ragg, H., and Weissman, C. (1985) *Cell* 41, 497–507.
Saito, I., Oya, Y., and Shimojo, H. (1986) *J. Virol.* 58, 554–560.
Saito, I., Oya, Y., Yamamoto, K., Yuasa, T., and Shimojo, H. (1985) *J. Virol.* 54, 711–719.
Sam, C.-K., and Dumbell, K.R. (1981) *Ann. Virol.* (Inst. Pasteur) 132E, 135–150.
Sambrook, J., Rogers, L., White, J., and Gething, M.-J. (1985) *EMBO J.* 4, 91–103.
Samulski, R.J., Berns, K.I., Tan, M., and Muzyczka, N. (1982) *Proc. Natl. Acad. Sci.* 79, 2077–2080.
Sandri-Goldin, R.M., Goldin, A.L., Levine, M., and Glorioso, J.C. (1981) *Mol. Cell. Biol.* 1, 743–752.
Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, P.M. (1981) *Mol. Cell. Biol.* 1, 486–496.
Sassone-Corsi, P., Wildeman, A., and Chambon, P. (1985) *Nature* 313, 458–463.
Sato, K., Ito, R., Back, K.-H., and Agarwal, K. (1986) *Mol. Cell. Biol.* 6, 1032–1043.
Schaefer-Ridder, M., Wang, Y., and Hofschneider, P.H. (1982) *Science* 215, 166–168.
Schaffner, W. (1980) *Proc. Natl. Acad. Sci.* 77, 2163–2167.
Schöler, H.R., and Gruss, P. (1984) *Cell* 36, 403–411.
Schöler, H., Haslinger, A., Heguy, A., Holtgreve, H., and Karin, M. (1986) *Science* 232, 76–80.

Simonsen, C.C., and Levinson, A.D. (1983) *Proc. Natl. Acad. Sci.* 80, 2495–2499.
Smith, G.L., Mackett, M., and Moss. B. (1983a) *Nature* 302, 490–495.
Smith, G.L., and Moss. B. (1983) *Gene* 25, 21–28.
Smith, G.L., Murphy, B.R., and Moss. B. (1983b) *Proc. Natl. Acad. Sci.* 80, 7155–7159.
Sodroski, J.G., Rosen, C.A., and Haseltinc, W.A. (1984) *Science* 225, 381–385.
Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S.Z., Popovic, M., Arga, S., Gallo, R.C., Haseltine, W.A. (1985) *Science* 227, 171–173.
Solnick, D. (1981) *Cell* 24, 135–144.
Southern, P.J., and Berg, P. (1982) *J. Mol. Appl. Genet.* 1, 327–341.
Stafford, J., and Queen, C. (1983) *Nature* 306, 77–79.
Stark, G.R., and Wahl, G.M. (1984) *Ann. Rev. Biol.* 53, 447–492.
Stow, N. (1981) *J. Virol.* 37, 171–180.
Stow, N.D. (1982) *Nucl. Acids Res.* 10, 5105–5119.
Stow, N.D., and Wilkie, N.M. (1976) *J. Gen. Virol.* 33, 447–458.
Subramani, S., and Southern, P.J. (1983) *Anal. Biochem.* 135, 1–15.
Sussman, D.J., and Milman, G. (1984) *Mol. Cell. Biol.* 4, 1641–1643.
Thomas, K.R., Folger, K.R., and Capecchi, M.R. (1986) *Cell* 44, 419–428.
Thummel, C., Tjian, R., and Grodzicker, T. (1981) *Cell* 23, 825–836.
Thummel, C., Tjian, R., and Grodzicker, T. (1982) *J. Mol. Appl. Genet.* 1, 435–446.
Thummel, C., Tjian, R., Hu, S.L., and Grodzicker, T. (1983) *Cell* 33, 455–464.
Tooze, J. (1981) *Molecular Biology of Tumor Viruses.* Part 2. *DNA Tumor Viruses*, 2d ed., Cold Spring Harbor Laboratory, New York.
Tratschin, J.-D., West, M.H.P., Sandbank, T., and Carter, B.J. (1984) *Mol. Cell. Biol.* 4, 2072–2081.
Treisman, R. (1985) *Cell* 42, 889–902.
Van Doren, K., Hanahan, D., and Gluzman, Y. (1984) *J. Virol.* 50, 606–614.
Venkatesan, S., Baroudy, B.M., and Moss, B. (1981) *Cell* 25, 805–813.
Venkatesan, S., Gershowitz, A., and Moss, B. (1982) *J. Virol.* 44, 637–646.
Villarreal, L.P., and Soo, N.J. (1985) *J. Mol. Appl. Genet.* 3, 62–71.
Wang, Y., Stratowa, C., Schaefer-Ridder, M., Doehmer, J., and Hofschneider, P.H. (1983) *Mol. Cell. Biol.* 3, 1032–1039.
Weintraub, H., Izant, J.G., and Harland, R.M. (1985) *Trends Genet.* 1, 1–22.
Weir, J.P., and Moss. B. (1983) *J. Virol.* 46, 530–537.
Wiktor, T.J., Macfarlan, R.I., Reagan, K.J., Dietzschold, B., Curtis, P.J., Wunner, N.H., Kieny, M.P., Lathe, R., Lecocq, J.-P., Mackett, M., Moss, B., and Koprowski, H. (1984) *Proc. Natl. Acad. Sci.* 81, 7194–7198.
Yamada, M., Lewis, J.A., and Grodzicker, T. (1985) *Proc. Natl. Acad. Sci.* 82, 3567–3571.
Yewdell, J.W., Bennink, J.R., Smith, G.L., and Moss, B. (1985) *Proc. Natl. Acad. Sci.* 82, 1785–1789.

Vectors

A Survey of Molecular Cloning Vectors and Their Uses

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CONTENTS

EXHIBIT C

2 Antibody Structure and Function

There are three sets of molecules involved in immune recognition: antibodies or immunoglobulins (Ig), T cell receptors (TCRs) and MHC molecules. Of these, antibodies were the first to be identified as serum molecules capable of neutralizing a number of infectious organisms.

Antibodies are produced by B cells in two distinct forms, either as cell surface receptors for antigen or, if the B cell is stimulated to differentiate into an antibody-forming cell (AFC), as soluble antibodies in extracellular fluids. It was the availability of large amounts of secreted antibodies which led to the early elucidation of their primary and tertiary structures. This showed that antibodies were formed from a series of globular domains, each with distinct functions. Such structural motifs have since been found in a very wide range of molecules involved in immune recognition and cell–cell interaction: these are products of the Ig supergene family. Although Igs themselves are extremely structurally diverse, the essential role of secreted antibody is to act as a bifunctional molecule (adapter) which can bind to antigen via specific antigen-combining sites and then crosslink the antigen to cells of the immune system or activate the complement system.

ANTIBODY STRUCTURE

Domains

The basic building block of all antibodies is the four polypeptide chain unit, consisting of two identical light chains and two identical heavy chains, crosslinked by interchain disulphide bonds and stabilized by non-covalent interactions (see Chapter 1). Light chains (κ or λ) are folded into two globular domains while heavy chains comprise four or five domains, depending on the class of the antibody — IgM and IgE have five heavy chain domains and IgG, IgA and IgD have four.

The N-terminal domains of both light and heavy chains are highly variable between antibodies (V domains), while the remaining domains are relatively constant (C domains), although they do differ between isotypes. The domains themselves consist of a β-barrel, with seven (C domains) or nine (V domains) strands of β sheet, stabilized by an intrachain disulphide bond. Within the V domains, the variability is clustered in three hypervariable regions, which are formed by amino acid residues 30–36, 50–65 and 93–102 (approximately). Although separated in the primary sequence, the hypervariable regions are brought together by the folding of the V domain and appear as loops at the tip of the molecule. The association of a heavy chain V domain and a light chain V domain therefore generates a surface with six exposed hypervariable loops, three from each chain. This surface forms the antigen binding site (paratope).

The precise sequence of the six hypervariable loops determines the antibody specificity, as may be demonstrated by transferring sets of hypervariable regions from one antibody to another by genetic engineering. Because of their role in forming the antigen binding site, the hypervariable regions are sometimes called the complementarity determining regions (CDRs) and by the same token the surrounding sequences, which form the β-barrel, are termed framework regions

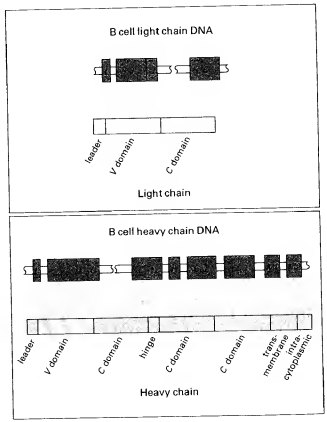

Fig. 2.1 The relationship of antibody genes to their polypeptides. In a mature B cell the gene encoding the expressed light chain consists of three exons: an *L* exon encoding a leader peptide required for translation of the polypeptide across the membrane of the endoplasmic reticulum, an exon formed by the recombination of a *V* and a *J* gene, which will encode the variable domain, and a *C* exon encoding the constant domain. Heavy chains in these cells (here illustrated as membrane IgG1) also have a leader. The variable domain is encoded by an exon formed by the recombination of three separate gene segments, that is a *V* gene, a *D* gene and a *J* gene. There are separate exons for each constant domain, one for the hinge (H) and two for the transmembrane (M1) and intracytoplasmic portions (M2) of the membrane antibody.

The C domains of an antibody confer the overall Y-shape of the molecule, and those of the heavy chain determine the antibody's class and subclass (these are the different Ig isotypes). The C-terminal domains of the heavy chains, which form the Fc region, are particularly important in determining the biological functions of the antibody. The process of antibody formation within an individual B cell allows genes for different heavy chain isotypes (C_H) to become associated with the cell's functional heavy chain variable region (V_H) gene. This means that a cell can generate antibody of a particular specificity, but with different functions, depending on which isotype(s) is used.

Individual Ig domains interact with their neighbours, so for example, the V_H and V_L domains interact extensively via the 3-strand β-sheet layers, while $C_\gamma 1$ and C_L make contacts between the 4-strand layer; residues along these interacting faces are predominantly hydrophobic. The hinge region between C_H2 and C_H3 is also important in conferring some segmental flexibility on the molecule and this is correlated with length of the hinge region in different subclasses — in mouse, IgG2b > IgG2a > IgG1.

The two forms of antibody, membrane and secreted Ig, differ only at the C-terminus where membrane Ig has transmembrane and short intracytoplasmic regions (3–30 residues depending on the subclass). The gene/exon organization of antibodies faithfully mirrors the structural subdivision of the polypeptide chains (Fig. 2.1).

Isotypes

In man there are nine functional heavy chain isotypes corresponding to the Ig subclasses and these may be associated with either of the two light chain isotypes κ or λ (see Fig. 2.9). In mouse there are eight heavy chain isotypes, but 90% of the serum antibody has κ light chains. The mouse λ chain locus has only a limited capacity to generate diversity, although there are four λ isotypes.

Antibody function is largely determined by the specificity and affinity of the antigen binding site and by the heavy chain isotype. Different subclasses vary in their ability to bind to cellular Fc receptors, complement, and transport receptors (Fig. 2.2). In practice, IgG, IgA and IgE antibodies have higher antigen binding affinities than IgM, but this is related to the affinity maturation which occurs concurrently with class switching in B cells, and is quite unrelated to the C domains (see Chapter 10). The essential characteristics of the different subclasses of serum antibodies may be summarized as follows:

Properties					Function (features common to humans)							Human		Mouse	
	Subunits	H chain domains	Size	Serum half-life (human) days	Complement fixation	Platelet binding	Mast cell and basophil sensitization	Transplacental transfer	Transepithelial transfer	Opsonization for macrophages, neutrophils, eosinophils	Sensitization for K cells	Binding to lymphocytes	Subclasses	Serum concentration mg/ml	Subclasses	Serum concentration mg/ml
IgM	5	5	19s	5.1	++	–	–	–	(+)	–	–	T‡	μ	1.5	μ	0.6-1.0*
IgG	1	4	7s	23*	+*	+	–	+*	(+)	+*	+*	T‡B	γ_1	9	γ_1	4.6-6.5
													γ_2	3	γ_{2a}	1.0-4.2
													γ_3	1	γ_{2b}	1.2-2.0
													γ_4	0.5	γ_3	0.1-0.2
IgA	1,2 or more	4	7s or 11s	5.8	–	–	–	–	++	–	–	T‡B‡	α_1	3	α	0.26-0.4
													α_2	0.5		
IgD	1	4	7s	2.8	–	–	–	–	–	–	–	–	δ	0.03	δ	–‡
IgE	1	5	8s	2.3	–	–	++	–	–	–	–	T‡B‡	ε	0.05×10^{-3}	ε	0.1×10^{-3}

*The range of mouse subclasses varies greatly between strains and at different ages

• Some subclasses only
‡ Some subsets of cells only

‡Serum levels of mouse IgD are very low or undetectable

Fig. 2.2 Characteristics and functions of antibody classes. Antibodies of different classes are formed from one or more basic subunits of two heavy and two light chains. The heavy chain determines the antibody class; the number of domains in the heavy chain may be 4 or 5, depending on the class, one of which is variable and the others constant. Antibody effector functions relate largely to the cells and effector systems with which the Fc regions interact. Complement, platelets, mast cells and basophils mediate inflammatory responses. Macrophages and neutrophils phagocytose and destroy antigenic particles opsonized by antibody. Eosinophils and K cells engage target cells via antibody bound to Fc receptors, while the receptors on T and B cells are involved in immunoregulation (see Fig. 2.14).

- **IgM** – first to be produced in a primary response; low affinity but high avidity to multivalent antigens; complement fixing.
- **IgG** – the main serum antibody, particularly in secondary response; essential in protection of neonate by transplacental transfer or in milk; has the ability to bind many cell types and to fix complement (varies with subclass).
- **IgA** – the main isotype secreted across mucosal surfaces.
- **IgE** – triggers inflammatory reactions via specific receptors on mast cells and basophils; elicits effector responses to some gut parasites.
- **IgD** – primarily a cell surface receptor, expression varies during B cell differentiation.

The Antigen Binding Site

Initial crystallographic analysis of antigen–antibody complexes used myeloma antibodies bound to small haptens such as vitamin K or phosphorylcholine (PC). These suggested that the haptens occupied small clefts on the antibody, and were bound by 5–10 non-covalent interactions. For example, the antibody TE33, specific for a peptide of cholera toxin, has a hydrophobic pocket lined by two tyrosines, two tryptophans, one histidine and one phenylalanine residue, which interact with the antigen.

More recently, the structures of a number of complexes between antibodies and intact protein antigens have been defined. These have clarified the nature of the interactions and show that antibody contacts large antigens over an extended area of surface shape-complementarity (about 750Å^2), equivalent to about 30% of the total CDR-determined paratope surface. In these cases there are no obvious antigen binding clefts, but the opposing surfaces of the antigen and antibody are in such close proximity that water must be almost totally excluded from the interface. In different antibodies there are 14–19 contact residues, and similar numbers on the antigen (Fig. 2.3). In the examples studied to date, every one of the hypervariable loops made some contribution to the binding site, and in some instances framework residues contribute to the binding (see colour figure 1).

Contact residues on antigens. Many techniques have been used to examine how different residues on an antigen contribute towards its epitopes. In principle, one can envisage an epitope consisting of a single linear sequence of polypeptide (continuous epitope) or one formed from several different regions of the primary sequence, brought together in the tertiary structure (discontinuous epitope). In practice, the size of the antigen combining site means that it will comfortably accommodate more than a single segment of polypeptide and so the great majority of antibodies which bind intact antigen have been shown to contact discontinuous epitopes.

One technique for mapping contact residues on antigens has employed species-specific variants of the antigen that differ by a small number of amino acids. This approach has been used for myoglobin and lysozyme, amongst others. If an antibody binds to one variant but not to another, then the failure to bind can be attributed to those amino acids which differ (Fig. 2.4). Unfortunately, this technique is limited by the availability of suitable variants. For example, when the binding of antibody HyHEL-10 to lysozyme was examined crystallographically, it was found that it bound regions around the active site of the enzyme. For obvious functional reasons, these areas are highly conserved between different lysozymes, and so they had not been identified as forming the centre of the epitope. The other limitation of this approach is the assumption that an amino acid difference will alter the binding affinity of the antibody: while this is generally true, it is sometimes too small to be readily detected. Site-directed mutagenesis now allows more accurate identification of the contact residues on both antigen and antibody.

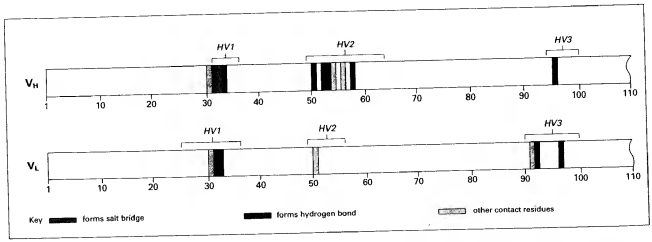

Fig. 2.3 Antigen contact residues on antibody to lysozyme. The amino acid residues from the antibody HyHEL-10 which make contact with a discontinuous epitope on hen eggwhite lysozyme are illustrated. Residues from both the V_H (upper) and V_L domains contact the antigen. Residues in green make hydrogen bonds with the antigen, and the single residue in grey forms a salt bridge. The relationship of these residues to the hypervariable regions is illustrated. Note that one of the contact residues in V_H lies in the first framework segment. Based on data from Padlan *et al.*, 1989. See also colour figure 1.

It appears that virtually the entire surface of a protein is potentially antigenic. The three antibodies which have been crystallized in complexes with lysozyme contact three epitopes with virtually no overlap, covering nearly 50% of the surface of the antigen; this suggests that with enough antibodies available, the entire surface could form epitopes (see colour figure 1). Nevertheless, some areas of antigens are more immunogenic than others, and these are often in regions of greatest molecular mobility. It is thought that these areas will tolerate more distortion as the antigen–antibody bond is formed, and are therefore more able to make a complementary fit to the paratope. This distortion is termed 'induced fit' (see below).

Molecular interactions. The bonds between an epitope and paratope include hydrophobic interactions, Van der Waal's forces, hydrogen bonds and ionic bonds. It is particularly notable that charged residues on the antigen are frequently neutralized by oppositely charged residues on the antibody. For example, in HyHEL-5 (anti-lysozyme) there are two adjoining glutamic acid side chains lying at the base of a groove, which neutralize the charge of a pair of protruding arginine residues on lysozyme. Charge neutralization is particularly important at the centre of the binding region; around the periphery, charged residues are not always neutralized, since they are partially accessible to water. The anti-lysozyme antibody, HyHEL-10 and its antigen provide a typical example of the types of interactions involved in an antigen–antibody complex. In this case there are 14 hydrogen bonds, 111 Van der Waal's contacts and 1 salt bridge.

In some cases, comparison of the crystallographic structures of complexed and uncomplexed molecules has shown that the interaction between antigen and antibody can induce changes in the molecular structure of either partner. Changes in the overall secondary structures are slight, but there may be considerable alterations in the orientation of individual side chains. For example, Trp-62 in lysozyme rotates through 150° about the C^{β}–C^{γ} bond in order to avoid steric hindrance from a tyrosine residue on the HyHEL-10 antibody. Presumably the degree of antigen distortion allowed

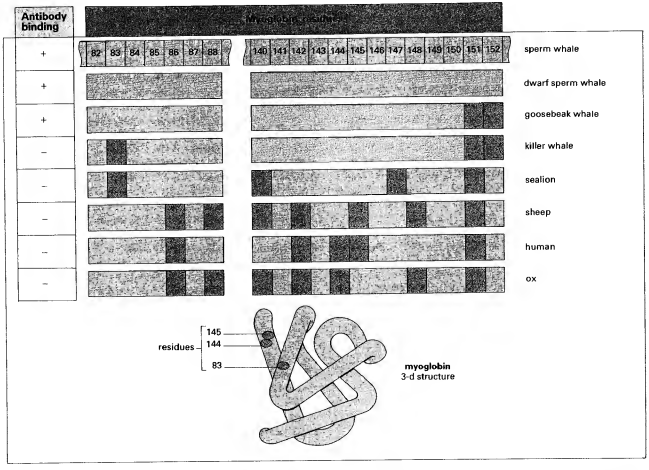

Fig. 2.4 Identification of contact residues on an epitope of myoglobin. A monoclonal antibody raised to sperm whale myoglobin was tested for its ability to bind myoglobins from other species (left column). Two regions on the antigen were identified as being important for the binding. The diagram indicates where the myoglobins differ from the sequence in sperm whale (yellow). It is found that a difference at residue 83 or 144 or 145 prevents antibody binding, indicating that these residues (at least) are involved in the interaction. These residues are clustered together in the tertiary structure of intact myoglobin (lower diagram). Based on data of Benjamin *et al.*, 1984.

depends on the degree of local flexibility. Similar arguments apply to changes in the antibody where it has been shown that hypervariable loops may undergo positional modifications to facilitate binding.

Idiotopes. Idiotopes are antigenic determinants associated with an antibody's binding site which may be recognized by other antibodies. Before detailed structures of antibodies became available, anti-idiotypic antibodies were the only way of distinguishing particular molecular shapes of the antigen binding site and associated V regions. Individual antibodies could be identified by their set of idiotopes which are said to determine the idiotype of an antibody. Some idiotopes were detected on many different antibodies (cross-reactive, recurrent or public idiotopes), while others were apparently unique to a single clonotypic antibody (private idiotope). In some cases, anti-idiotypic antibodies could block the binding of antigen to the cognate antibody implying that the idiotope was associated with the antigen binding site (site-associated idiotope). Other anti-idiotopes appeared to recognize structures outside the binding site.

Experiments in mice have shown that certain idiotypes are characteristic of the immune response to specific antigens in particular strains. For example, in C57BL/6 mice, the primary antibody response is dominated by antibodies expressing the T15 idiotype. Evidence has accumulated to show that immunoglobulin heavy and light chain haplotypes are the critical factors which determine whether one of these 'germline idiotypes' may be produced by a particular strain. Other studies have shown that antibodies from genetically different individuals, directed to the same antigen, can also share particular idiotypes. This has been described for a number of human autoantibodies. In such cases, it appears that the constraint of binding to a particular epitope leads to the generation of antibodies with similarly shaped paratopes.

Recent advances have made it possible to equate the serological and genetic data on idiotypes with the precise structures of the V domains. These suggest that a typical idiotope will be similar to an epitope on a conventional protein antigen. For example, a study of the major cross-reactive idiotype produced in A/J mice on *p*-azobenzene arsonate antibodies showed that both light chain and heavy chain residues were required for anti-idiotype binding. Light chains alone were not recognized by the anti-idiotype, but heavy chains of the correct type would bind 20–35% of the antibodies in a polyclonal anti-idiotype, even if they were associated with an incorrect light chain. In this case, the idiotopic site appeared to include the D region of the heavy chain (within the third hypervariable region) and one or both of the other V_H CDRs.

The crossreactive idiotope IdX, which occurs on antibodies to dextran (only in mice of the IgH^a haplotype), has been studied in some detail. The IdX-bearing antibodies can be generated by two or more related V_H segments in association with a number of different D segments. The IdX idiotope appears to depend on two residues (Asn–Asn) in the second hypervariable region. Two IdX-bearing antibodies, M104 and J558, differ by only two amino acid residues in the D segment, but can still be distinguished by anti-idiotypic antibodies (see colour figure 2.). The M104 private idiotope corresponds with Tyr–Asp or Ala–Asp residues in their D segments, while the J558 idiotope is expressed by antibodies having Arg–Tyr at these positions. This example illustrates how very similar antibodies can express both public and private idiotopes.

Comparison of idiotopes in different strains has confirmed the association with particular haplotypes and genes. For example, the T15 idiotype, mentioned above, is expressed on a heavy chain assembled from V_H1 of the *S107* gene family, recombined with $D_HFL16.1$ and J_H1, and associated with a κ light chain using the $V_\kappa22$ germline gene. The idiotope AB1–2 on this antibody depends on the third heavy chain hypervariable loop (D-region), but is absent from IgH^j strains. This absence is correlated with an allelic form of $D_HFL16.1$ in IgH^j mice which can be detected by RFLP analysis.

These analyses are not just of academic interest, since particular genes are selectively used for production of certain autoantibodies. For example, the κ *V* region which encodes an anti-DNA antibody in NZB × NZW and MRL lpr/lpr mice has not yet been detected in antibodies to any external (non-self) antigens. In addition, T cells control the level of expression of some idiotypes during an immune response; this control mechanism is discussed in Chapter 12.

C Domains

C domains and the hinge region are involved in the interactions of antibody with complement C1q and Fc receptors.

In IgG, the two C_H3 domains are paired with a contact area of approximately 1000Å^2, but the C_H2 domains are separated, with carbohydrate units lying in the cleft between them. The carbohydrate units may be one of about 20 different structures based on a mannosyl-chitobiose core. They confer some resistance to proteolysis, and also affect the rate of catabolism *in vivo*. Although the carbohydrate does not form elements directly responsible for C1q or Fc receptor binding, it does maintain the conformation of the Fc region, so that it can bind these receptors. For example, carbohydrate-deficient IgG1 and IgG3 antibodies to the hapten NIP, bound very weakly to $Fc_\gamma RI$ and $Fc_\gamma RIII$, although there was little conformational change to the Fc regions, as judged by their continued ability to bind Fc-specific monoclonal antibodies.

Fc receptor binding and complement activation. Complement C1q binds to a region in the C_H2 domain of IgG with a binding affinity (K_a) of approximately 10^6M^{-1}. Complexed IgG makes a much higher avidity bond to C1q because of the multivalent interaction between the complex and C1q, and not because of any conformational change in the C_H2 domain. Different subclasses vary in their ability to bind C1q and activate complement (see Chapter 15), but binding alone is not the only factor which determines whether a subclass is active. For example isolated Fc fragments of IgG4 bind C1q, but the intact molecules do not, due to steric hindrance from the Fab arms. In addition, complement C3b and C4b often bind to the C_H1 domains of IgG, and so activation efficiency may also depend on the availability of suitable

binding sites in this domain.

There are three major types of Fc_γ receptor and two Fc_ε receptors in man (see below). Binding affinity of monomeric IgG to $Fc_\gamma RI$ is high ($K_a = 5.5 \times 10^8 M^{-1}$) with IgG1 and IgG3 binding more strongly than IgG4, and IgG2 being inactive. The other two Fc_γ receptors are of intermediate affinity ($<10^6 M^{-1}$), and therefore only bind immune complexes effectively. The binding site for $Fc_\gamma RI$ is close to the hinge in the linking region to the C_H2 domain. This was demonstrated by site-directed mutagenesis in which the affinity of a mouse IgG2b for this receptor was increased 100-fold by substituting Glu for Leu 325. The site for $Fc_\gamma RII$ appears to be in C_H2 while that for $Fc_\gamma RIII$ requires both C_H2 and C_H3.

Polymeric Immunoglobulins

Mammalian IgM is a pentamer or hexamer of the basic four chain Ig unit, and in most mammals (man is an exception) serum IgA is polymeric, consisting mainly of dimers. Polymerization of these classes is dependent on the J (joining) chain (which has no relationship whatsoever to the *J* gene segments which contribute to the V domain -OH or $-NH_2$ groups).

The J chain consists of 137 amino acid residues in both man and mouse, encoded by four exons with 77% homology between the two sequences. One model of the J chain suggests that it consists of a single domain with a β-barrel structure similar to Ig domains, although it is not a member of the Ig supergene family. However, crystallographic analysis of J chain structure is still awaited.

The J chain assists polymerization by crosslinking cysteine residues, with disulphide bonds, in the C-terminal domains of secreted IgM and IgA; these are absent in the membrane forms. Evidence for the role of the J chain in polymerization comes from somatic cell hybrids of IgG-producing myelomas (also producing J chain) fused with a B cell lymphoma producing monomeric IgM. These hybrid cells were able to assemble pentameric IgM. The actual polymerization is probably effected by a sulfhydryl oxidase, found exclusively in fully differentiated antibody forming cells. J chain is critical for facilitating IgM polymerization, although polymers lacking a J chain can still be secreted. Strangely, even cells producing IgG exclusively, synthesize considerable quantities of J chain. The function in these cells (if any) is unknown, and it is broken down internally.

Dimeric IgA can bind to the poly-Ig receptor on the serosal side of glandular epithelia (e.g. in the gut) to be transported across to the luminal face. The poly-Ig receptor, a 5-domain member of the Ig supergene family, is then cleaved releasing the IgA and leaving part of the cleaved receptor to form the secretory piece, which wraps around the C-terminal domains of IgA and protects it from digestion (Fig. 2.5). Binding to the poly-Ig receptor is dependent on the presence of a J chain, since artificial polymers lacking J chain bind poorly. The production of these secreted Igs is essential in the protection of mucosal surfaces.

Allotypic Variants

The immunoglobulins of individuals of a species express allotypic determinants that can be recognized by other individuals lacking the allotype. Human Ig allotypes were originally identified by sera of patients with rheumatoid arthritis which contain naturally occurring anti-Ig antibodies; but anti-allotypic antibodies raised by deliberate or accidental immunization are usually more specific for single allotypes. Five sets of allotypic markers have been described in humans. These are the Gm, Am and Mm systems which are determined by the heavy chain C regions, the Km (=Inv) system determined by the κ genes, and the Hv system determined by the V_H genes (which could be considered a public idiotype marker). Since heavy chains of different classes may combine with κ chains the Km allotypes may occur in association with any of an individual's heavy chain allotypes on different antibodies (Fig. 2.6 overleaf).

The Gm series of heavy chain allotypic determinants, occurring on IgG constant regions are the most numerous (at least 24 variants). Most of these allotypic sites have been localized to the Fc region, but G1m(4) and G1m(17) are determined by the C_H1 domain. Since the anti-allotype sera recognize determinants rather than individual isotypes, a few allotypic markers, called isoallotypes, can occur on different subclasses, although most are expressed on only one subclass. To indicate

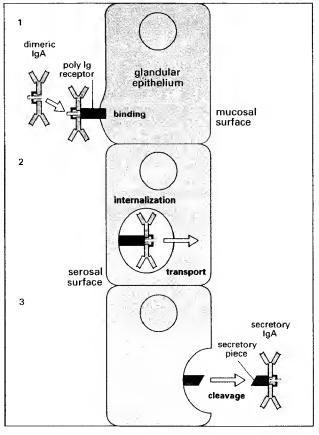

Fig. 2.5 Secretion of IgA. Dimeric IgA can link to the poly-Ig receptor exposed on the serosal side of glandular epithelia (e.g. the gut). The IgA/receptor complex is internalized and transported to the mucosal surface where it is released by proteolysis. The ligand binding portion of the receptor remains attached to the IgA dimer by disulphide bonds, and is in fact the secretory piece, which protects IgA from degradation in the mucosal secretions.

this, the nomenclature includes a figure showing which subclasses each allotype is associated with. For example G1m(4) and G1m(17) are associated with IgG1, while G3m(5) and G3m(6) occur on IgG3. An example of an isoallotype is IgG4 nG4m(a) which is also present on all IgG1 and IgG3 molecules. In some cases, if an individual haplotype carries one Gm allotype it precludes the presence of another (e.g. G3m(5) and G3m(21)), which implies that the allotypes are determined by close or identical amino acids. Sometimes, the presence of the allotype depends on single amino acid changes. For example, G1m(4) has Arg at position 214 of the γ1 chain while IgG1 antibodies lacking this allotype do not. In other cases the determinant is produced by the three-dimensional structure of the Ig. For example, the Km light chain determinants are only weakly expressed on free light chains, or on κ chains associated with γ2 or γ4 heavy chains.

Km (= Inv)	κ chains	expressed on IgG1 and IgG3; 3 variants; requires IgG quarternary structure for expression
Fd	heavy chain V domain	one variant
Am	Fc_{α}	two variants of IgA2
Mm	Fc_{μ}	one variant
Gm	Fc_{γ} and C_H1	at least 24 variants 3 associated with IgG1 1 with IgG2; 13 with IgG3 + 7 unassigned

Fig. 2.6 Human Ig allotypes. This table lists five major systems of human Ig allotypes, and their gene locations.

Although Ig allotypes are of interest to population geneticists, one might wonder whether they have any functional significance. There have been reports of disease association with particular allotypes (e.g. rheumatoid arthritis), but this may only reflect a tighter linkage to V region idiotypes and the B cell repertoire. In fact, the associations are relatively weak when compared with MHC-related disease associations. However, it has been noted that the levels of particular isotypes within an individual are partly dependent on that individual's allotype. For example, individuals who are homozygous for G3m(5) have approximately twice the amount of serum IgG3 than individuals who lack the allotype. Heterozygotes have intermediate levels.

Ig allotypes are present in every animal species tested so far. In mice they have been very useful for determining the origin of secreted Ig in experiments where donor cells of one allotype are transferred into a recipient of another allotype. Unfortunately, the nomenclature of mouse Ig allotypes is extraordinarily confusing. The allotypes of the *Ig-1* gene locus encode IgG2a heavy chains, *Ig-2* encodes IgA, *Ig-3* encodes IgG2b, *Ig-4* encodes IgG1, *Ig-5* encodes IgD and *Ig-6* encodes IgM. There are between two and eight alleles for each of these loci, with greater numbers of recognized allotypic determinants. For example, the eight allotypes of *Ig-1* include ten recognized allotypic determinants. Again, it is doubtful whether this genetic complexity has any more than a marginal effect on the operation of the immune system.

GENE STRUCTURE AND EXPRESSION

The antigen binding domains of Igs are generated during B cell ontogeny by a process of somatic recombination between different gene segments. A very similar process occurs during T cell development in the thymus. The basic principles of the recombination will be described here, while the ways in which this process leads to the generation of Ig and T cell receptor (TCR) diversity is examined in Chapter 4.

There are three separate loci encoding Ig chains, namely the heavy chain locus (IgH) and those for the κ and λ light chains. These loci undergo recombination at two separate stages during B cell development, as evidenced by changes in the DNA-restriction enzyme-generated fragments of the Ig genes in mature B cells. The first stage involves the recombination of *V*, *D* and *J* segments to form the gene encoding the V_H and V_L domains. This takes place during differentiation of lymphoid stem cells into virgin B cells. The second type of gene recombination may occur in the C_H genes of differentiated B cells, and is involved in irreversible class switching by the B cell.

Recombination of *V*, *D* and *J* Genes

The heavy chain V domain gene is assembled from a *V* gene encoding approximately the first 94 residues, which combines with a *D* gene segment (diversity) and a *J* gene segment (joining). The region of the *V*–*D*–*J* junction forms the third hypervariable region of the V domain, while the first and second hypervariable regions are encoded within the *V* gene. There are more than 200 *V* genes in the IgH locus, with 10 *D* genes and 4 *J* genes. Since any of the *V* genes can recombine with any *D* gene and any *J* gene, the number of possible combinations of *V*–*D*–*J* is enormous (see Chapter 4). Light chain genes also undergo recombination, but these loci only contain *V* and *J* gene segments, so the third hypervariable region of the light chain is formed at a *V*–*J* junction. Recombination of either heavy or light chain genes leads to the loss of the intervening stretches of DNA, containing both introns and exons (Fig. 2.7).

Recombination takes place in a defined sequence; heavy chains first. The cell attempts to make a functional recombination from one of its chromosomes, and if this fails, it turns to the other. The production of a functional

ADVANCED IMMUNOLOGY

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